

NORADRENERGIC CONTROL OF SPINAL MOTOR
CIRCUITRY IN TWO RELATED AMPHIBIAN SPECIES
'XENOPUS LAEVIS' AND 'RAMA TEMPORARIA'

Jonathan R. McDermid

A Thesis Submitted for the Degree of PhD
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**Noradrenergic control of spinal motor
circuitry in two related amphibian
species: *Xenopus laevis* and *Rana
temporaria*.**

A thesis submitted to the University of St. Andrews for the degree of
Doctor of Philosophy.

by
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July 1998



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Declaration

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This thesis is dedicated to all my friends and family, and to the memory of Lyle Henry.

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Abbreviations

5HT	5-hydroxytryptamine
ACh	acetylcholine
AD	adrenaline
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APV	5-amino phosphonvaleric acid
α -BTX	α -bungarotoxin
COMT	catechol-O-methyl-transferase
CNS	central nervous system
CPG	central pattern generator
DA	dopamine
DHK	dihydrokainic acid
dla	dorsolateral ascending
dlc	dorsolateral commissural
EAA	excitatory amino acid
EDRF	endothelium derived relaxing factor
epsp	excitatory postsynaptic potential
GABA	gamma aminobutyric acid
HCG	human chorionic gonadotrophin
ipsp	inhibitory postsynaptic potential
(s)ipsp	spontaneous ipsp
KAc	potassium acetate
KCl	potassium chloride
L-DOPA	L-dihydroxyphenylalanine
L-NOARG	N ^G -nitro-L-arginine

MAO	monoamine oxidase
mhr	mid-hindbrain reticulospinal
MLR	mesencephalic locomotor region
MSR	monosynaptic reflex
NA	noradrenaline
NADPH	nicotinamide adenosine dinucleotide phosphate
NMDA	N-methyl-d-aspartate
NO	nitric oxide
PB	phosphate buffer
PDA	piperidinedicarboxylic acid
PNS	peripheral nervous system
PKA	protein kinase A
R-B	Rohon-Beard
SCN	suprachiasmatic nucleus
SNAP	S-nitroso-n-acetyl-penicillamine
STNS	stomatogastric nervous system
TH	tyrosine hydroxylase
TTX	tetrodotoxin

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Abstract.

1. The role of the catecholamine noradrenaline (NA) was examined during fictive swimming in *Xenopus laevis* tadpoles.
2. The primary effects of the amine in both embryonic and larval *Xenopus* was to markedly decrease motor frequency whilst simultaneously reducing rostrocaudal delays during swimming.
3. The NA-mediated modulation of swimming activity in *Xenopus* larvae can be reversed with phentolamine, a non-selective α adrenergic receptor antagonist, suggesting that NA may be acting through either α_1 or α_2 receptors, or a combination of both.
4. Intracellular recordings made from embryo spinal motoneurons revealed that reciprocal inhibitory glycinergic potentials are enhanced by NA. This effect is most prominent in caudal regions of the spinal cord where inhibitory synaptic drive is generally weaker.
5. NA was also found to enhance glycinergic reciprocal inhibition during swimming in larval spinal cord motoneurons.
6. Intracellular recordings, under tetrodotoxin, reveal that NA enhances the occurrence of spontaneous glycinergic inhibitory post synaptic potentials arising from the terminals of inhibitory interneurons, suggesting that the amine is acting presynaptically to enhance evoked release of glycine during swimming.

7. The effects of NA on swimming frequency and rostrocaudal delay appear to be largely mediated through an enhancement of glycinergic reciprocal inhibition as blockade of glycine receptors with strychnine weakens the ability of the amine affect these parameters of motor output.

8. The effects of NA on motor output were also examined in embryos of the amphibian *Rana temporaria*. Whilst NA did not obviously affect swimming activity, the amine induced a non-rhythmic pattern of motor activity.

9. The free radical gas, nitric oxide also induced a non-rhythmic pattern of motor discharge that was remarkably similar to that elicited by NA, indicating that this neural messenger may be important for motor control.

CHAPTER 1

General Introduction

i) Background.

The way in which neurones are organised to form networks and communicate to generate appropriate behaviours is a fundamental question that has yet to be fully answered. Less than two decades ago, neurones were thought of as having fixed, hard-wired properties and it was generally accepted that summation of their synaptic inputs was the major means of determining the output of neural networks. However, great advances in our understanding of this field have been made in recent years and this concept is now widely disregarded. It has now been shown that both the biophysical membrane properties of neurones and the way in which they interact synaptically is fluid and subject to neuromodulation by various transmitters and peptides. These endogenous substances exert a wide range of effects on the integrative and electrical properties of neurones which have now been characterised in some detail at the cellular level (Kaczmarek & Levitan, 1987). However, less is known of the wider significance of these findings with respect to neuromodulation at the network level in adult vertebrates. This is largely because the immense complexity of nervous systems has made it difficult to examine the cellular and synaptic mechanisms that underlie their behaviour. With regard to this problem, simpler invertebrate models have proven more successful in revealing how behaviours are generated, perhaps the most notable of which is the crustacean stomatogastric nervous system (STNS). The STNS, which is responsible for driving several different but functionally related motor patterns in the foregut of these animals, has not only provided valuable insights into how behaviour is generated, but also into how it is made flexible through neuromodulation. A wide range of chemical modulators have been shown to target the STNS and their effects on the ganglia of this network have now been characterised at both the cellular and network level (for review, see Harris-Warrick, Nagy & Nusbaum, 1992). Studies into the

effects of these modulators has shown that, not only can they modify the expression of ongoing motor programmes, but they can also reconfigure the network so that it is capable of generating several different types of output.

Although the crustacean STNS, along with other invertebrate nervous systems (such as those of molluscs, annelids and insects; see Dickenson, 1989 and Harris-Warrick & Marder, 1991 for reviews), have played a key role in developing understanding of neural circuitry and its modulation, they do have limitations with respect to understanding vertebrate nervous systems. Due to their complexity, there are very few vertebrate models that have proven useful as tools for studying motor behaviour. Notable exceptions are the adult lamprey and the *Xenopus* embryo preparations (for reviews see Roberts 1990 and Grillner & Matsushima, 1991). The remarkably simple nervous system of the stage 37/8 *Xenopus* embryo is capable of generating relatively stereotyped yet well co-ordinated swimming activity that makes it an ideal preparation for studying the neural basis of a vertebrate motor behaviour. The cellular and synaptic basis of swimming activity in this animal has been examined in considerable detail and the three types of neurone involved in rhythm generation have been identified (Roberts & Clarke, 1982; Dale, 1985; Dale & Roberts, 1985). The way these neurones connect to form a functional network has been deduced (Dale, 1985; Dale & Roberts, 1985; Perrins & Roberts, 1994c). Also, many of the biophysical membrane properties of each class of rhythm generating neurone have been described (Soffe, 1990; Dale, 1995a). The *Xenopus* embryo preparation is currently one of the best understood models for vertebrate behaviour available, providing a solid basis from which to study the effects of neuromodulation within a neural network. More recently, the *Xenopus* embryo preparation has been used as a tool for examining the postembryonic ontogeny of motor circuitry subsequent to the time of hatching. Over a brief period in development, between embryonic stage 37 and larval stage 42, a marked increase in the complexity of motor output

during swimming occurs (Sillar, Wedderburn & Simmers, 1991). The cellular and synaptic changes that occur during this transition from relatively simple to more complex behaviour are beginning to be revealed (Sillar, Wedderburn & Simmers, 1992; Sun & Dale, 1998).

Investigations into the generation of swimming in the *Xenopus* embryo have highlighted a number of basic principles which may be applicable to vertebrate behaviours in general. The use of other vertebrate preparations is therefore important to establish whether this is the case. Indeed, several other amphibian embryo preparations have been studied with this in mind (Soffe, Clarke & Roberts, 1984). The late embryo of the frog *Rana temporaria* (stage 20, Gosner, 1960; Soffe, 1991a) is one such example. Compared to the *Xenopus* embryo, this species expresses a more 'adult-like' motor pattern during swimming in that ventral root output in this preparation is more bursty and occurs at lower frequency than that of the *Xenopus* embryo (Soffe, 1991a). Therefore this preparation has proven useful as a tool for examining how more complex behaviours are generated. Recent work examining the neural networks that generate swimming in *Xenopus* and *Rana* has provided an insight into how differences in synaptic drive, membrane properties and neuromodulatory inputs determine the type of behaviour that is expressed (e.g. Perrins & Roberts, 1995a; Perrins & Soffe, 1996a; Perrins & Soffe, 1996b; Sillar, Wedderburn & Simmers, 1992; Sillar, Woolston & Wedderburn, 1992; Soffe & Perrins, 1997; Soffe & Sillar, 1991).

Both the *Xenopus* and *Rana* preparations are potentially useful tools for the study of how neuromodulators affect vertebrate motor behaviour. Noradrenaline (NA) is one such modulator that has been shown to influence motor output in several animals (e.g. Barbeau & Rossignol, 1991; McPherson & Kemnitz, 1994) although its effects on motor output in *Xenopus* and *Rana* are unknown. My goals were to investigate the effects of NA on motor activity in

these animals and establish the mechanisms through which this important but poorly understood amine acts. The remainder of this chapter will provide a more detailed resume of the *Xenopus* and *Rana* preparations: Firstly, in the next section, the composition and function of the neural networks that generate swimming behaviour in the *Xenopus* embryo will be outlined. This will be followed by a brief account of how the *Xenopus* preparation has proven useful in examining the role of one particular brainstem neuromodulator, 5-hydroxytryptamine (5-HT) in the ontogeny and modulation of swimming behaviour. Finally, current knowledge on *Rana* embryo swimming and the neural networks that underlie this behaviour will be addressed.

ii) The *Xenopus* embryo preparation.

Close to the time of hatching, at around two days after fertilisation, the embryo of the South African clawed frog *Xenopus laevis* (stage 37/8, Nieuwkoop & Faber, 1956; figure 1.1A) is capable of generating sustained episodes of swimming activity following sensory stimulation (such as touching of the trunk or tail skin, or, less reliably, in response to dimming of illumination). This swimming activity occurs at a velocity of around 5cm.s^{-1} and is characterised by waves of myotomal muscle contractions that alternate across the body and progress rostrocaudally at around 15cm.s^{-1} (Roberts, 1990, Figure 1.1B). Episodes of swimming can last for up to several minutes and either terminate spontaneously or when the animal contacts an obstruction in its path.

When paralysed in the acetylcholine nicotinic receptor antagonist α -bungarotoxin (α -BTX) the discharge of motoneurone axons that innervate the myotomal muscle blocks can be recorded using glass suction electrodes placed over the intermyotomal clefts. Under these conditions, brief (0.5-1ms) electrical

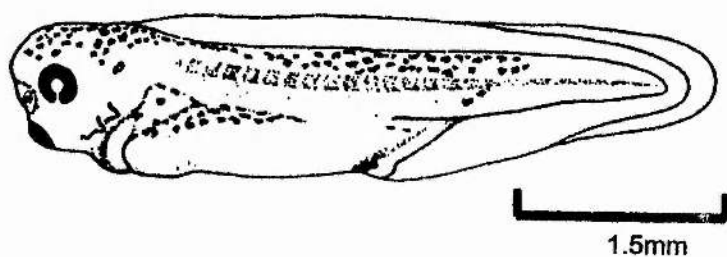
Figure 1.1. Swimming in the *Xenopus* embryo.

A. Close to the time of hatching from its egg membrane, the stage 37/8 embryo is around 5mm in length.

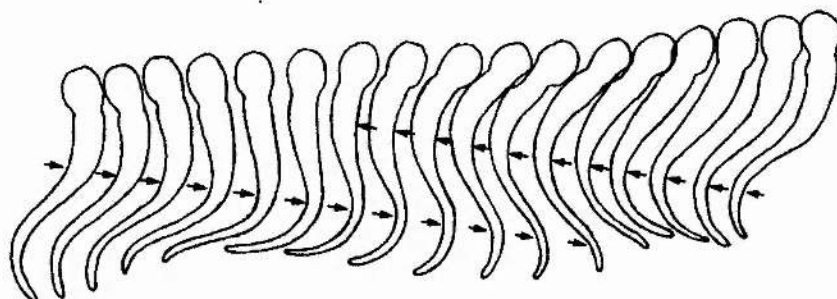
B. The animal swims at frequencies of 10-20Hz by generating lateral undulations which pass first down one side of the body and then the other. Arrows denote points of maximum body curvature. Drawing from Kahn & Roberts, 1982b.

C. When paralysed in the neuromuscular blocking agent α -bungarotoxin, ventral root activity can be recorded using glass suction electrodes placed over the intermyotomal clefts. This activity alternates between the right and left sides of the body (depicted by dotted lines between R5 and L5) whilst passing down each side with a brief intermyotomal delay (depicted by dotted lined between L5 and L13) and as such is entirely appropriate for generating real swimming behaviour. In this example, three recordings have been made. One from the 5th post otic intermyotomal cleft on the right side of the body (R5), one from the 5th intermyotomal cleft on the left side of the body (L5) and one from the more caudal 13th intermyotomal cleft on the left side of the body (L13).

A



B



C



current pulses applied to the trunk or tail skin initiate bouts of rhythmic ventral root activity suitable to drive swimming behaviour. This activity is characterised by strictly alternating waves of ventral root discharge that progress rostrocaudally with a brief delay between each cleft (figure 1.1C). Such behaviour is termed 'fictive swimming' and occurs at frequencies of 10-20Hz. Since no proprioceptive input is generated during fictive swimming (Kahn & Roberts, 1982a), the activity must be programmed centrally without a requirement for sensory feedback. Furthermore, essentially identical fictive swimming activity occurs when descending spinal influences are removed by transection of the spinal cord at the level of the first post-otic cleft, suggesting that the neural elements essential for generating swimming behaviour are intrinsic to the spinal cord (Roberts, Soffe & Dale, 1986).

iii) Neuroanatomy of the *Xenopus* embryo spinal cord.

Initial attempts to classify different types of neurone in the embryo spinal cord according to anatomical criteria employed the horseradish peroxidase (HRP) staining technique (Roberts & Clarke, 1982). This study demonstrated that only eight anatomically distinct classes of neurone are present in the spinal cord at this stage in development. The schematic in figure 1.2 gives an impression of the spatial location of each of these types of neurone. A synopsis of the morphology of each cell type is given below.

Three types of neurone involved in the processing of skin sensory information are present in the *Xenopus* embryo spinal cord. Of these, the Rohon-Beard (R-B) cells are primary mechanosensory neurones that respond to mechanical deformation of the skin. They have relatively large cell bodies located in the medial aspect of the dorsal horn from which usually only a single,

Figure 1.2. *Xenopus* spinal cord neuroanatomy.

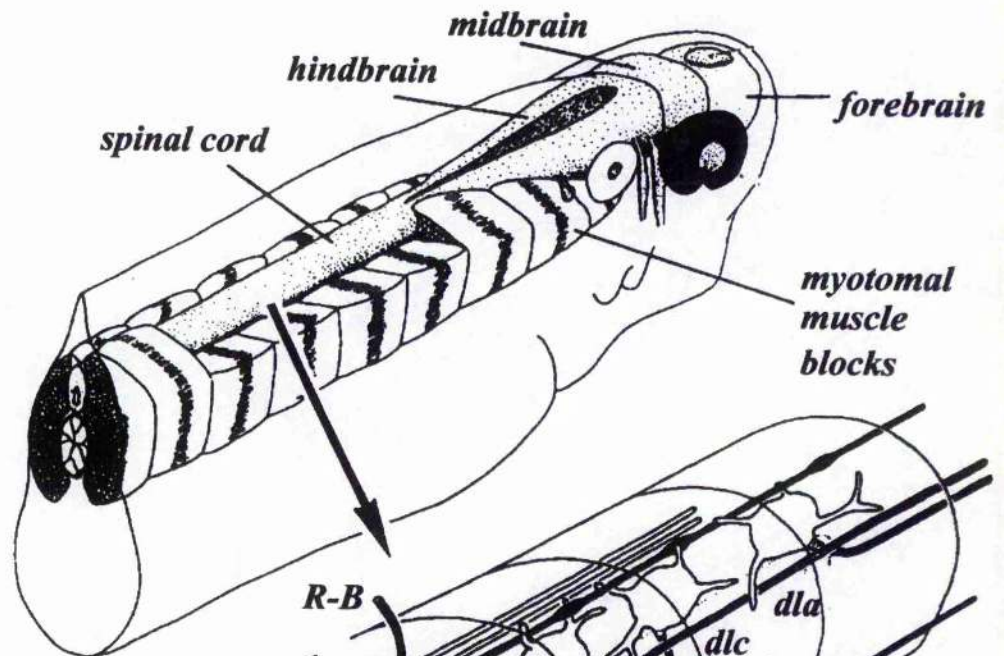
A. The spinal cord is a gently tapering tube that is enveloped by the myotomal muscle blocks. The embryo CNS is comprised of the forebrain, midbrain, hindbrain and spinal cord.

B Diagrammatic representation of the spatial location of each of the three classes of sensory neurone present within the embryo spinal cord. These are the Rohon Beard cells (**R-B**) the ascending dorsolateral sensory interneurons (**dla**) and the commissural dorsolateral sensory interneurons (**dlc**). The Kölmer-Agdhur cells, whose function is unknown are also illustrated (**K-A**).

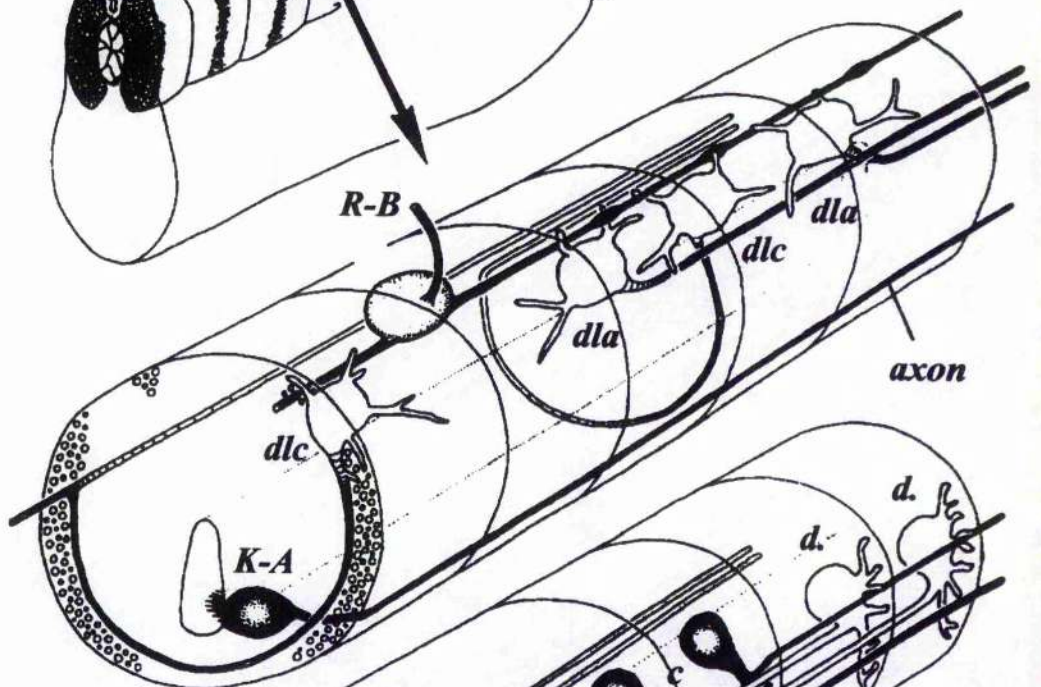
C. Diagrammatic representation of the spatial location of each class of neurone in the spinal motor system. These are the descending interneurons (**d**), the ascending interneurons (**a**), the commissural interneurons (**c**) and the motoneurons (**mn**).

Figure adapted from Roberts, 1990.

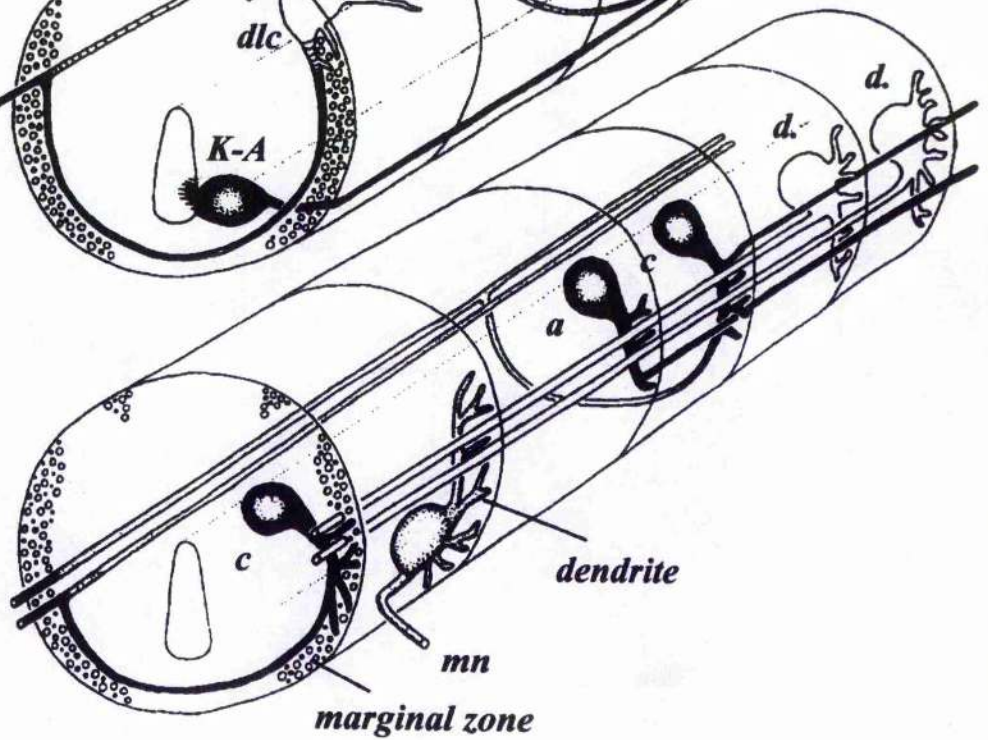
A



B



C



unmyelinated neurite extends to innervate the skin (Hughes, 1957; Roberts & Hayes, 1977). The central axons of these cells bifurcate to ascend as far as the hindbrain and also descend within the spinal cord. Located just beneath the R-B cells, at the dorsal limit of the marginal zone are two classes of second-order sensory interneurone. These are the dorsolateral ascending (dla) and the dorsolateral commissural (dlc) interneurons. Dendrites from both classes of dl interneurone branch out to receive synaptic contact from R-B cells in the dorsal tract. The axons of dla neurones appear to ascend ipsilaterally in the lateral tract whereas the axons of dlc neurones cross to the opposite side of the spinal cord where they both descend and ascend in the lateral tract.

Between the dorsal and ventral regions, at an intermediate level, three anatomically distinct classes of interneurone are found: the commissural interneurons, the descending interneurons and the ascending interneurons. The commissural interneurons have unipolar somata from which there extends one primary neurite that runs ventrally along the inside edge of the lateral tract to branch out into short radial dendrites. The axons of these cells cross to the opposite side of the spinal cord where they extend caudally as well as rostrally towards the level of the hindbrain. Descending interneurons have multipolar somata that are found on the inside border of the lateral tract and their dendrites extend ipsilaterally in the lateral and dorsal tracts. Their axons course in a caudal direction in the lateral tract just dorsal to the motoneurons. The ascending interneurons have unipolar somata that are found deep within the spinal cord relative to the lateral tract. A single process extends into the inner margin of this tract where dendrites branch out. The axons of these cells lie in the inner part of the lateral tract where they ascend to the hindbrain and also descend for a short distance.

Spinal motoneurons have their cell bodies in the ventral part of the spinal cord, along the inner edge of the lateral tract. Motoneurone somata extend

down the spinal cord in columns which can often be more than one layer thick. Dendrites of these cells innervate the lateral tract whilst their axons commonly extend in a caudal direction, usually as far as the next intermyotomal cleft before turning obliquely to exit the spinal cord and innervate the myotomal muscle blocks. Some of the motoneurones possess two peripheral axons which innervate more than one motor nerve.

The final type of cell found in the embryo spinal cord are the Kölmer Agdhur cells. These ciliated ependymal cells have also been described in many vertebrate species. They contact the neurocoel and have axons which run in the lateral tract to extend as far as the hindbrain. The cells are immunoreactive for GABA (Dale, Roberts, Ottersen & Storm-Mathisen, 1987) but their function is currently unknown.

iv) Rhythm generation in the embryo spinal cord.

Electrophysiological experiments suggest that of the eight spinal cell types, only the motoneurones, the commissural interneurones and the descending interneurones are involved in locomotor rhythm generation. A brief review of the role these neurones in rhythm generation is given below.

Intracellular recordings from motoneurones have shown that the synaptic drive during swimming comprises three prominent components: i) phasic excitation that triggers action potentials on each cycle, ii) tonic excitation that generates the sustained level of depolarisation that occurs throughout an episode of swimming and iii) phasic inhibition which occurs in the middle of each cycle when neurones on the opposite side of the cord are active. A significant proportion of the phasic and all of the tonic excitatory drive is thought to arise from the activation of two different excitatory amino acid (EAA) receptor

subtypes. Unitary excitatory post synaptic potentials (epsps) evoked in motoneurons by extracellular stimulation of spinal cord axons comprise two components, distinguishable by their kinetics and pharmacology. Epsps that have a slow rise and slow fall time are blocked by the NMDA receptor antagonist (\pm)-2-amino-5-phosphonovaleric acid (APV) suggesting they are NMDA-receptor mediated. The remaining component has both a fast rise and fall time and is blocked by cis-2,3-piperidine dicarboxylic acid (PDA), an AMPA/NMDA receptor antagonist (Dale & Roberts, 1985). Both components appear to originate from the same population of neurones, the descending interneurons (Dale & Roberts, 1985). The NMDA receptor-mediated epsps have durations that are relatively long (around 200ms, Dale & Roberts, 1985) when compared to the cycle periods attained during embryo swimming (50 to 100ms). As such, they will summate from one cycle to the next to generate the sustained tonic depolarisation seen in the motoneurons during swimming. The shorter AMPA-mediated epsps are likely to generate a component of the phasic, on-cycle excitation during swimming.

Paired recording studies have shown that descending premotor interneurons (identified morphologically by subsequent HRP staining) artificially depolarised above threshold by current injection evoke dual-component epsps in motoneurons that have the same pharmacology as those generated by extracellular stimulation (Dale & Roberts, 1985). It is therefore likely that they are the source of excitatory drive during swimming. These descending interneurons are also presumed to mutually re-excite themselves as they receive the same synaptic drive during swimming as all other rhythmically active neurones. Computer models have suggested that this positive-feedback mutual re-excitation may be essential for sustained rhythm generation (Roberts & Tunstall, 1990).

More recent reports have shown that as little as 30% of the fast 'on-cycle' synaptic excitation seen in motoneurones during fictive swimming is mediated by EAA transmission. The remainder appears to be generated by the motoneurones themselves (Perrins & Roberts, 1995a,b,c). Paired recording studies have shown that action potentials evoked in rostral motoneurones elicit one-for-one short latency epsps in more caudal motoneurones (Perrins & Roberts, 1995a). These epsps comprise both a cholinergic and an electrical component (Perrins & Roberts, 1995a). The cholinergic component occurs between motoneurones that are no more than 200 μ m apart and is blocked by mecamylamine and so is likely to be mediated by nicotinic receptors. Furthermore, local perfusion of another nicotinic receptor antagonist, dihydro- β -erythroidine, onto motoneurones indicates that cholinergic transmission accounts for around 20% of the phasic excitatory drive during swimming (Perrins & Roberts, 1995b). It appears that motoneurones may also form weaker cholinergic synapses with premotor interneurones (Perrins & Roberts, 1995c).

Local perfusion of cadmium (Cd^{2+}) onto motoneurones to block their chemical synaptic drive during fictive swimming only diminishes on-cycle excitation by around 50%. The remaining Cd^{2+} -insensitive component is likely to be generated by electrotonic coupling between motoneurones (Perrins & Roberts, 1995b). Electrical synapses are thought to be present between neighbouring motoneurones that are no more than 70 μ m apart (Perrins & Roberts, 1995a). Such connections are likely to enhance the reliability of motoneurone firing on each cycle of swimming activity and also help to synchronise firing among neighbouring motoneurones.

The final type of interneurone in the *Xenopus* embryo spinal cord is the commissural interneurone. The evidence outlined below suggests that these cells are responsible for generating the glycine-mediated reciprocal inhibition that couples the two sides of the spinal cord in antiphase during swimming. Spinal

motoneurones hyperpolarise when exposed to glycine, a response which becomes strongly depolarising when intracellular Cl^- levels are raised (Soffe, 1987). Two findings indicate that the source of endogenous glycine in the spinal cord of these animals is the commissural interneurone. Firstly, these are the only glycine immunoreactive neurones in the entire CNS of the embryo spinal cord (Dale, Ottersen, Roberts & Storm-Mathisen, 1986). Secondly, paired recording studies have demonstrated that these cells make short-latency, strychnine-sensitive monosynaptic inhibitory connections with neurones on the opposite (and in a small proportion of cells, the same) side of the spinal cord (Dale, 1985). All rhythmically active neurones -including motoneurones (Roberts & Kahn, 1982, Soffe & Roberts, 1982a), commissural interneurones (Soffe, Clarke & Roberts, 1984; Dale, 1985) and excitatory interneurones (Dale & Roberts, 1985)- receive inhibition 'mid-cycle' during swimming. The fact that this inhibition occurs mid-cycle can be explained by the observation that the commissural interneurones have axons which extend to the opposite side of the spinal cord. Therefore, impulses in commissural interneurones 'on-cycle' on one side of the spinal cord will generate inhibition 'mid-cycle' in cells on the other side and vice versa. A small proportion of commissural interneurones also have ipsilaterally projecting axons which synapse with neurones on the same side of the spinal cord (Dale, 1985). These projections are thought to generate on-cycle inhibition in neurones with which they synapse (Sillar & Roberts, 1992a; Sillar & Roberts, 1993; Perrins & Soffe, 1996a). The functional role of the commissural interneurones will be discussed in more detail in chapter 4.

v) The *Xenopus* preparation as a tool for the examination of the maturation of vertebrate locomotor networks.

The basic components of the neural networks that generate motor behaviour are often present at very early stages in vertebrate development and can be functional before birth or hatching. This is the case for animals such as arthropods (Cohen, Tamara, Dobrov, Keimel and Baker, 1990), fish (Batty, 1984; Kuwada, 1986), birds (Bekoff, 1992; O'Donovan, 1989) and mammals (Kudo & Yamada, 1987; Hernandez, Elbert & Droge, 1991). The human embryo, for example, can express rhythmic locomotor limb movements as early as 10 weeks in development (De Vries, Visser & Prechtl, 1984). Between these early stages in development and adulthood, profound changes in the CPGs for locomotion occur so that gradually, more complex and refined locomotor capability is acquired. The mechanisms involved in inducing this developmental maturation are not yet fully understood. Nonetheless, it would seem likely that the basic circuitry adequate to generate locomotor behaviour at early stages is retained and gradually adapted by the introduction of descending control and modulatory systems as development proceeds.

The *Xenopus* preparation has proven amenable for studying the ontogeny of vertebrate locomotor behaviour. Within a very short time period, between the embryonic stage 37/8 and the larval stage 42, profound developmentally-related changes in the motor capabilities of this animal occur. By stage 42 (Nieuwkoop & Faber, 1956), just 24 hours after hatching from their egg membranes, the simple biphasic ventral root activity characteristic of embryonic swimming is replaced by more complex and variable bursts of motor root discharge within each cycle (Sillar, Wedderburn & Simmers, 1991; see figure 1.3). The larvae also swim over a wider range of frequencies than the embryo (7 to 35 Hz c.f. 10 to 20 Hz in the embryo) and are capable of accelerating, decelerating and varying the intensity of

Figure 1.3. Developmental changes in swimming activity in *Xenopus* tadpoles.

A. At around two days after hatching, the *Xenopus* embryo is around 5mm in length (Ai) and capable of generating fictive swimming activity. Such activity when recorded from contralateral ventral root clefts is found to be relatively simple and stereotyped, comprising of single biphasic impulses of ventral root discharge on each cycle of activity (ii). Recordings using KAc-filled electrodes from motoneurons during swimming have shown that the synaptic drive that underlies this swimming activity is relatively basic and reliable, with motoneurons only firing a single impulse on each cycle of activity (iii).

B. Just 24 hrs later, at stage 42, the animal is now around 40% larger with a diminished yolk sack and a fully developed gut (Bi). Ventral root activity during fictive swimming at this stage is much more complex, so that it now consists of prolonged bursts of ventral root discharge (ii). This change in ventral root output is accompanied by a more complex synaptic drive to motoneurons in the stage 42 larvae so that they can now fire multiple spikes on each cycle of activity and can also drop out and be recruited back into the swimming network as required (iii).

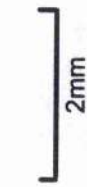
Figure adapted from Sillar, Reith & McDermid (In press).

Ai

Embryo

Bi

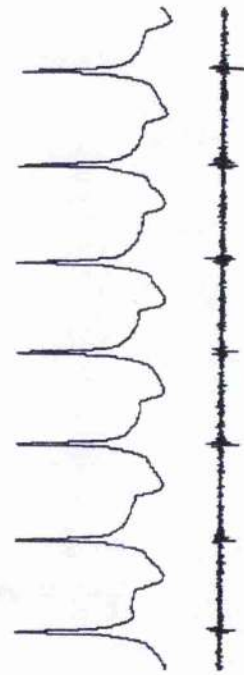
Larva



ii



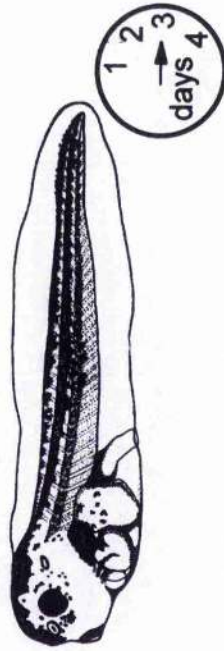
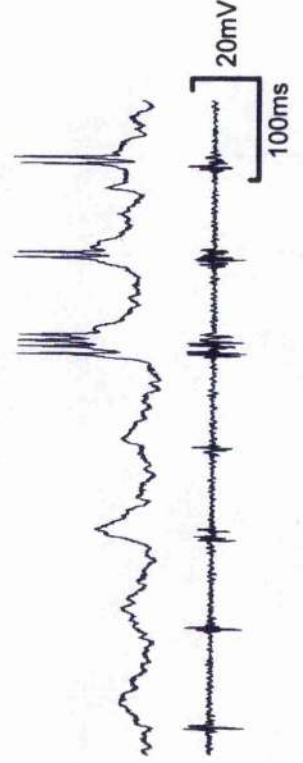
iii



ii



iii



motor output. Another difference that becomes apparent at this stage is that a relationship between rostrocaudal delay and cycle period appears in that these two parameters of swimming become positively correlated (Tunstall & Sillar, 1993). This contrasts with the situation in the embryo where rostrocaudal delays are relatively constant in duration, irrespective of swimming frequency (Dale & Soffe, 1991). Intracellular recordings made from larval motoneurons have shown that by developmental stage 42, the synaptic drive has changed profoundly so that it is now much more complex, an effect which is accompanied by the acquisition of multiple spiking capability within a single cycle of activity (figure 1.3, Sillar, Simmers & Wedderburn, 1992). This variability in synaptic drive and firing properties of spinal neurones presumably imparts a greater degree of flexibility to the larval motor pattern.

These differences in locomotor activity between the two stages of development has allowed the developmental processes that occur during ontogeny of the motor pattern to be studied. There is now considerable evidence to indicate that the brainstem neuromodulator 5-HT is responsible for imposing at least some of the changes in motor output seen during this period of development. Firstly, neuroanatomical evidence has shown that during a period of late embryonic and early larval development spanning stages 37 and 42, axons arising from serotonergic interneurons in the brainstem raphe nucleus gradually innervate the spinal cord (van Mier, Joosten, van Rheden & ten Donkelaar, 1986). Secondly, a wide range of physiological experiments have been performed that implicate 5-HT as being causal in the development of more complex motor activity. As stated earlier, in the stage 37/8 embryo, simple biphasic ventral root activity is apparent along the entire length of the animal and likewise, a more bursty pattern of motor output is obvious both rostrally and caudally at stage 42. However, between these stages, at stage 40 (around 12 hours post-hatching) rostral ventral root activity is bursty whilst caudal activity is more embryonic (Sillar, Wedderburn, & Simmers,

1991). When 5-HT is bath applied at any of these stages it produces motor output mimicking that expressed by the next stage of development. For example, 5-HT applied to embryos causes an increase in burst durations rostrally but not caudally, to produce motor output resembling that normally recorded in the stage 40 larva. Bath application of 5-HT to stage 40 larvae causes burst durations to increase in both rostral and caudal regions leading a swimming motor pattern reminiscent of the stage 42 larva. When 5-HT is bath applied to stage 42 larvae, the motor pattern is profoundly modulated in a manner similar to that of other vertebrates so that burst durations both rostrally and caudally are markedly increased. These effects can be reproduced with agonists and blocked by antagonists to 5-HT receptors (Wedderburn & Sillar, 1994). 5-hydroxytryptophan (5-HTP), the metabolic precursor to serotonin is also capable of mimicking the action of 5-HT. Finally, when the monoamine neurotoxin 5,7 dihydroxytryptamine is used to ablate aminergic projections early in development, the motor pattern remains embryonic at stage 42. This suggests that the acquisition of more complex motor behaviour is largely dependent upon serotonergic innervation of the spinal cord (Sillar, Woolston & Wedderburn, 1995). Bath application of 5-HT has also been shown to impose a relationship between swimming frequency and longitudinal delay in *Xenopus* embryos. In the larvae, where cycle periods scale with delays, the amine strengthens the correlation between these two parameters of swimming (Tunstall & Sillar, 1993).

The cellular and synaptic mechanisms through which 5-HT acts to induce more bursty, intense motor output have recently been investigated. Recordings from ventral motoneurons in the spinal cord have shown that 5-HT reduces the amplitude of the mid-cycle inhibitory component of fictive swimming an effect that may be mediated through a decrease in the presynaptic release of glycine from the terminals of commissural interneurons (McDearmid, Sillar & Wedderburn, 1997). This weakening of inhibitory synaptic strengths has been

suggested to lead to the enhanced firing of motoneurons because the phasic excitation is less likely to be terminated by weakened inhibition (McDearmid, Sillar & Wedderburn, 1997). However, other mechanisms may well be involved. More recently, a fast activated Ca^{2+} -dependant K^+ current which is absent in dissociated spinal motoneurons of the embryo is expressed in motoneurons of stage 42 larvae (Sun & Dale, 1998). Down-regulation of this current by iberiotoxin induces prolonged bursts of ventral root discharge similar to that seen in older animals in stage 41 and 42 but not stage 40 larvae. It remains to be seen if 5-HT is able to down-regulate this current, either through a direct action on this channel or an indirect action on calcium channels, thereby enhancing ventral root discharge in *Xenopus* larvae.

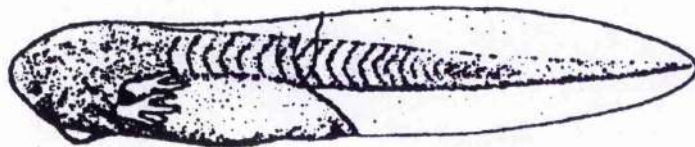
vi) Motor output in the Rana embryo.

Another, more recently developed amphibian preparation that is used for the study of vertebrate locomotor activity is the embryo of the frog *Rana temporaria* (figure 1.4A). At the late embryonic stage (stage 20, Gosner, 1960), close to their time of hatching, these embryos are at an equivalent point in development to the stage 37/8 *Xenopus* embryo. They express a range of both rhythmic and non-rhythmic motor behaviours, the fictive correlates of which can be recorded from the ventral root clefts after immobilisation in α -bungarotoxin (α -BTX). Fictive swimming in *Rana*, as in *Xenopus*, is characterised by waves of ventral root discharge which alternate across the body and progress rostrocaudally with a brief delay between myotomal muscle blocks (figure 1.4B). Bursts of ventral root output occupy around 40-50% of each cycle of swimming, with episodes commonly lasting only a few seconds. Swimming can either maintain a

Figure 1.4. Fictive motor output in *Rana* embryos.

The embryo of the frog *Rana temporaria* at stage 20 (A; Gosner, 1960; Soffe, 1991a) when paralysed in α -bungarotoxin is capable of generating fictive motor output that is suitable for driving swimming behaviour. This activity is characterised by waves of ventral root discharge that alternate between the two sides of the body and progress longitudinally down each side with a brief delay between myotomal muscle blocks (B). When recordings are made with KAc-filled microelectrodes from presumed motoneurons in the ventral quarter of the spinal cord, the synaptic drive to these cells during rhythmic motor activity is comprised of phasic excitation coincident with ventral root discharge followed by a period of hyperpolarising mid-cycle inhibition, superimposed on a level of tonic excitation (Ci). This inhibition is reversed in sign to become depolarising when KCl is used as the electrolyte (Cii). Dimming of illumination can cause the generation of a non-rhythmic motor response, which is accompanied by phasic depolarisations in motoneurons (Ciii).

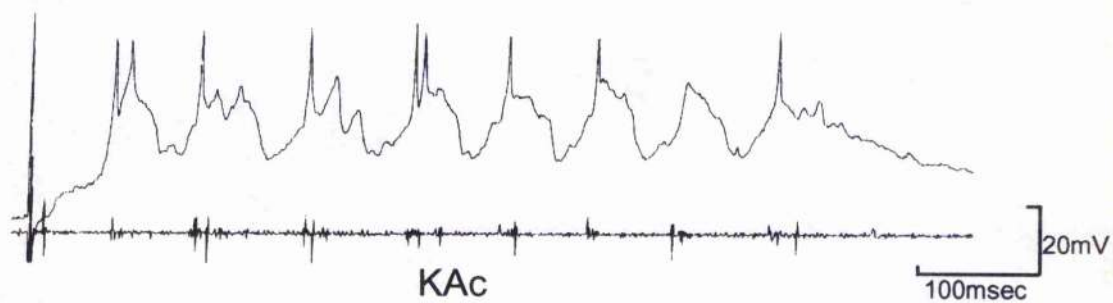
A



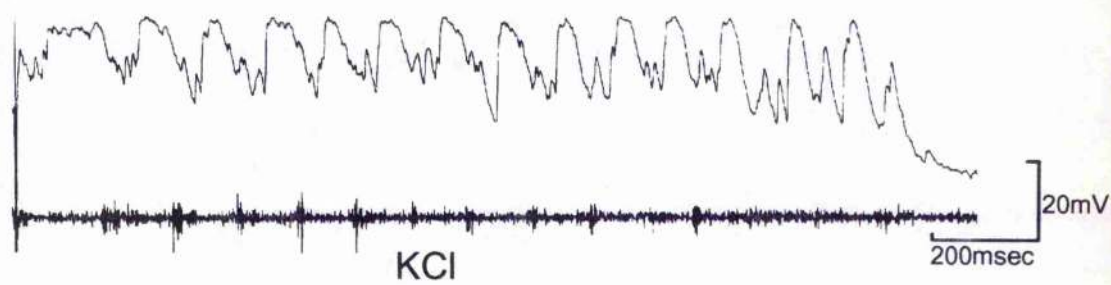
B



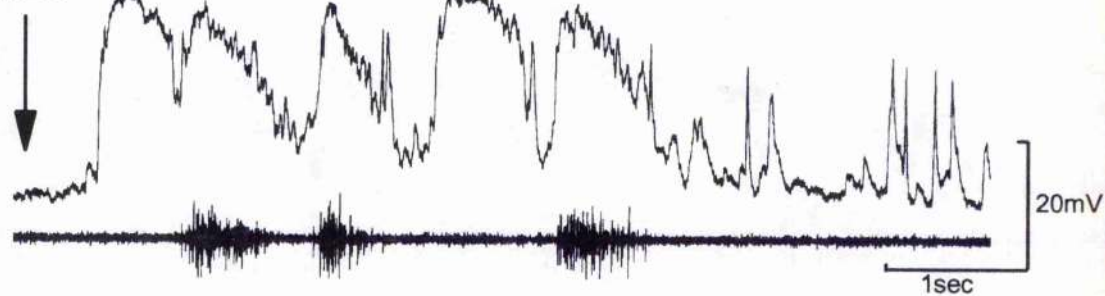
Ci



ii



iii
lights off



relatively constant frequency or can accelerate throughout an episode, sometimes reaching a plateau before rhythmic output is terminated (Soffe, 1991a).

Although very little is known of the neuroanatomy of the CPG that drives motor activity in *Rana* embryos, intracellular recordings from motoneurons have shown that the synaptic drive to these cells, although being much more complex than that of the *Xenopus* embryo, consists of the same three basic components: tonic and phasic excitation and hyperpolarising mid-cycle inhibition (figure 1.4Ci, Soffe & Sillar, 1991). Phasic excitation seems to be largely EAA and cholinergic in origin, although, unlike the *Xenopus* embryo, there appears to be no rhythmic electrical component during swimming (Perrins & Soffe, 1996b). Mid-cycle inhibition is both strychnine-sensitive and chloride-dependent (figure 1.4Cii, Perrins & Soffe, 1996b) and therefore presumably glycinergic. Less is known about the tonic excitatory component, although a proportion appears to be mediated by electrotonic synapses (Perrins & Soffe, 1996b). Hence, although there is currently no neuroanatomical evidence for the existence of glycine or glutamate immunoreactive commissural interneurons in the spinal cord of *Rana* embryos, it is reasonable to assume that the basic circuitry for locomotion is organised in a similar fashion to that of *Xenopus*. Therefore, it could be simplistically viewed as two half-centres coupled by reciprocal glycinergic inhibition.

Aside from sustained rhythmic swimming activity, paralysed *Rana* embryos can also display non-rhythmic motor patterns (Soffe, 1991a). These are expressed in response to mechanical deformation or electrical stimulation of the skin or sometimes to dimming of the illumination (figure 1.4Ciii, Soffe, 1991a; Soffe & Sillar, 1991). They vary in duration and intensity from short single ventral root spikes to longer bursts of discharge that can last up to two seconds. Such activity can occasionally be followed by ventral root discharge on the contralateral side. Recordings from presumed motoneurons in the ventral

quarter of the spinal cord have shown that this motor activity occurs coincident with phasic depolarisations in ipsilateral motoneurons and phasic inhibition in contralateral motoneurons, indicating that it is most likely mediated by the same half-centres that generate rhythmic motor activity (Soffe & Sillar, 1991).

The stage 20 *Rana* embryo is at an equivalent point in development to stage 37/8 *Xenopus* in that both embryos are close to their time of hatching. Furthermore, the synaptic drive in both animals appears to comprise the same basic elements and therefore the CPGs for locomotion are also likely to be organised in a similar fashion. However, as it occurs at a much lower frequency and involves much more intense and prolonged bursts of motor output, swimming in *Rana* is more similar to adult vertebrate behaviours than that of the *Xenopus* embryo (Sillar & Soffe, 1989). This raises some interesting questions as to the neural mechanisms that underlie the expression of two very distinct behaviours in these closely-related amphibians. One possibility is that a difference in the timetable of ion channel expression on motoneurons of the two animals underlies the differential expression of the two motor patterns. Another is that due to different developmental timetables, the networks underlying motor behaviours in these two animals could be differentially exposed to descending neuromodulatory influences. The types of neuromodulators present and their effect on motor output may also differ between these two animals. It has already been shown that at least one descending spinal neuromodulator is present at an earlier stage in development in *Rana*: by stage 20, extensive serotonergic innervation of the spinal cord is already present in *Rana* embryos (Woolston, Wedderburn & Sillar, 1994). This contrasts with the stage 37/8 *Xenopus* embryo where serotonergic innervation of the spinal cord is less well developed.

The *Rana* and *Xenopus* preparations therefore provide a useful comparison for studying the descending influence that transmitters and modulators impart on spinal motor networks. The effects of 5-HT have been at least partially

investigated in both systems (Sillar, Wedderburn & Simmers, 1992; Sillar, Woolston & Wedderburn, 1992). However, the effects of another amine, noradrenaline (NA), have not been previously examined. In the three results chapters that follow, I will show that NA is capable of exerting a profound influence on the motor behaviour in both animals. In chapter three I will describe the modulatory effects that this amine imposes on the swimming motor pattern in *Xenopus* embryos and larvae. In chapter four I will show that this effects of NA on motor behaviour in *Xenopus* may be mediated through a strengthening of mid-cycle inhibition. Finally, in chapter five I will show that NA does not obviously modulate *Rana* swimming but rather initiates a non-rhythmic motor pattern, a response which is also triggered by the free radical gas nitric oxide (NO). Possible links between NA and NO systems will be discussed.

CHAPTER 2

Materials and Methods

i) Animals.

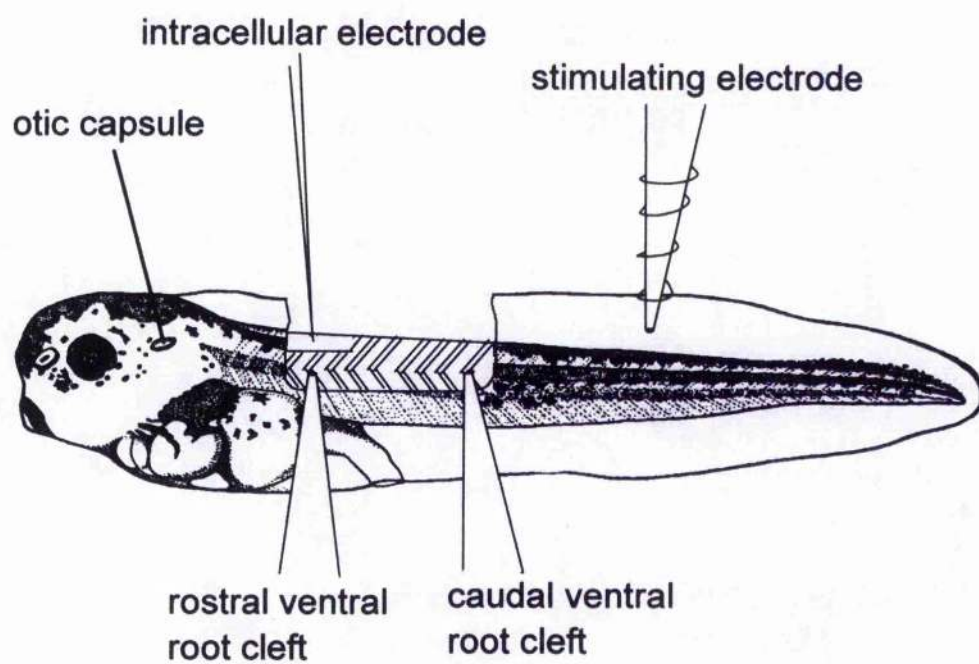
All experiments were performed on embryonic (stage 37/8), early larval (stage 42) *Xenopus laevis* and embryonic (stage 20) *Rana temporaria* tadpoles. Animals were staged according to the normal tables for *Xenopus laevis* (Neiwkoop & Faber, 1956) and *Rana* (Gosner, 1960). *Xenopus laevis* tadpoles were obtained through induced breeding by injection of human chorionic gonadotrophin (1000 units per ml, Sigma) into pairs of adult *Xenopus* obtained from a laboratory colony. Females were injected with 0.35ml and males with 0.15ml human chorionic gonadotrophin (HCG). Eggs were raised in aerated and dechlorinated tap water at 17-23°C until they had reached the desired experimental stage. *Rana temporaria* spawn, which is only available between the months of February and April, was ordered from Blades Biological or collected from local ponds. Eggs were raised in pond water at temperatures of 8-23°C until they were of the correct experimental stage.

ii) Experimental preparations and electrophysiological techniques.

The procedure for dissection of both stage 37/8 and 42 *Xenopus* tadpoles and stage 20 *Rana* embryos was essentially identical. An example of a stage 42 preparation is shown in figure 2.1. After having their tail skin slashed along the dorsal fin with fine etched tungsten dissecting needles to facilitate drug access, animals were placed in a chamber containing 2mls of the nicotinic acetylcholine receptor antagonist α -bungarotoxin (1.25 μ M, Sigma). Once complete neuromuscular block had occurred, the immobilised animals were removed from the α -bungarotoxin and placed in an experimental chamber (volume ca. 2ml) through which 100ml of frog Ringer solution (composition in mM: 115 NaCl, 2.5

Figure 2.1. The preparation.

The animal (a stage 42 *Xenopus* larva in this case), after paralysis in α -bungarotoxin, is pinned to a sylgard surface in the experimental chamber. The flank skin of the animal is removed from around the level of the first post otic myotome to the level of the anus. Glass suction electrodes are placed over the ventral root clefts to allow extracellular recordings to be made at both rostral and more caudal levels of the body. After clearing away a region of the muscle blocks, intracellular recordings were made from rhythmic neurones positioned in the ventral quarter of the spinal cord using fine microelectrodes. Glass stimulating electrodes placed on the tail skin of the animal are used to initiate bouts of motor activity.



KCl, 1 MgCl₂, 2.4 NaHCO₃, 10 HEPES, CaCl₂ 2 [extracellular experiments], 4 [intracellular experiments]), buffered to pH 7.4 with 1N NaOH, was continuously recirculated at room temperature. The saline was gravity fed from a stock bottle containing 100ml saline. Animals were then pinned out on their right sides through the notocord using fine etched tungsten pins on a rotateable Sylgard-coated platform within the experimental chamber.

In order to expose the myotomal muscle blocks which lie under the trunk and tail skin, fine forceps and mounted etched tungsten needles were used to remove a patch of skin from the otic capsule to around the mid trunk level. The impulse activity of motor axons in the ventral roots was recorded extracellularly by placing glass suction electrodes over the intermyotomal clefts. The clefts recorded from were numbered rostrocaudally from the level of the otic capsule. The electrodes were made from glass fiberless capillary tubes (1mm outer diameter, Clarke Electromedical Instruments) which were heated over a mini-bunsen and hand pulled to produce a tapered shaft. The glass was then cut with a diamond knife to produce a tip opening of ca. 50µm. Ventral root activity was amplified (x10K) using an AM-Systems differential amplifier (model 1700). During this study, a series of experiments were also performed on animals transected at the level of the first post-otic cleft to remove input from higher centres. Spinalisations were performed using fine dissecting pins. Following this procedure animals were left to recover for at least 15 minutes.

To enable intracellular recordings of motoneurone activity, tungsten needles were used to remove an appropriate region of myotomes and expose the underlying spinal cord. Neurones were then penetrated with glass microelectrodes pulled on a Campden Instruments moving coil microelectrode puller (model 753) from filamented glass capillary tubes (outer diameter 1mm). Microelectrodes were filled with 3M KCl and had DC resistances between 100-150MΩ. KCl was used as the electrolyte during the course of this study because it causes Cl⁻

leakage into the cell thereby making inhibitory Cl^- -mediated potentials strongly depolarising. Since I studied the effects of NA on spinal inhibitory transmission, the use of KCl as an electrolyte makes analysis of inhibitory events much easier. Recordings were made from cells in the ventral quarter of the spinal cord, where motoneurons are known to predominate (Roberts and Clarke, 1982). It was therefore assumed that the vast majority of rhythmically active cells recorded in this area were motoneurons. Penetrations were achieved by using capacity overcompensation. Intracellular signals were amplified (x10) with a laboratory made amplifier. All signals were converted to digital format and stored on a Vetter integrated video cassette format instrumentation recorder (model 420) and displayed on a Gould digital oscilloscope (model 1602). A Yokogawa oscillographic recorder (model ORP 1200), graphtec Thermal Arraycorder and Gould Colorwriter (model 6120) were used to make permanent records off-line.

Throughout experiments, fictive motor activity was evoked via either dimming of illumination (Roberts 1978) or by applying a 1ms current pulse to the tail skin (Clarke, 1984) via a stimulation suction electrode attached to a Digitimer DS2 isolated stimulator.

Drugs were bath applied by adding known quantities to the stock bottle to achieve the desired final bath concentration. The drugs used in this study are detailed below.

<i>Drug</i>	<i>Concentration (μM)</i>	<i>Source</i>
Alprenolol	20-200	RBI
Noradrenaline	1-10	Sigma/RBI
Phentolamine	20-100	RBI
Clonidine	1-100	Sigma
Phenylephrine	5-50	Sigma
Isoprenaline	5-100	Sigma
Strychnine	1	Sigma
Tetrodotoxin	0.5-1	Sigma
n-acetyl-penacillamine	100	RBI
SNAP	100	Chemistry department St. Andrews University.
L-NOARG	100	RBI
5-hydroxytryptamine	1-10	Sigma

iii) Data analysis.

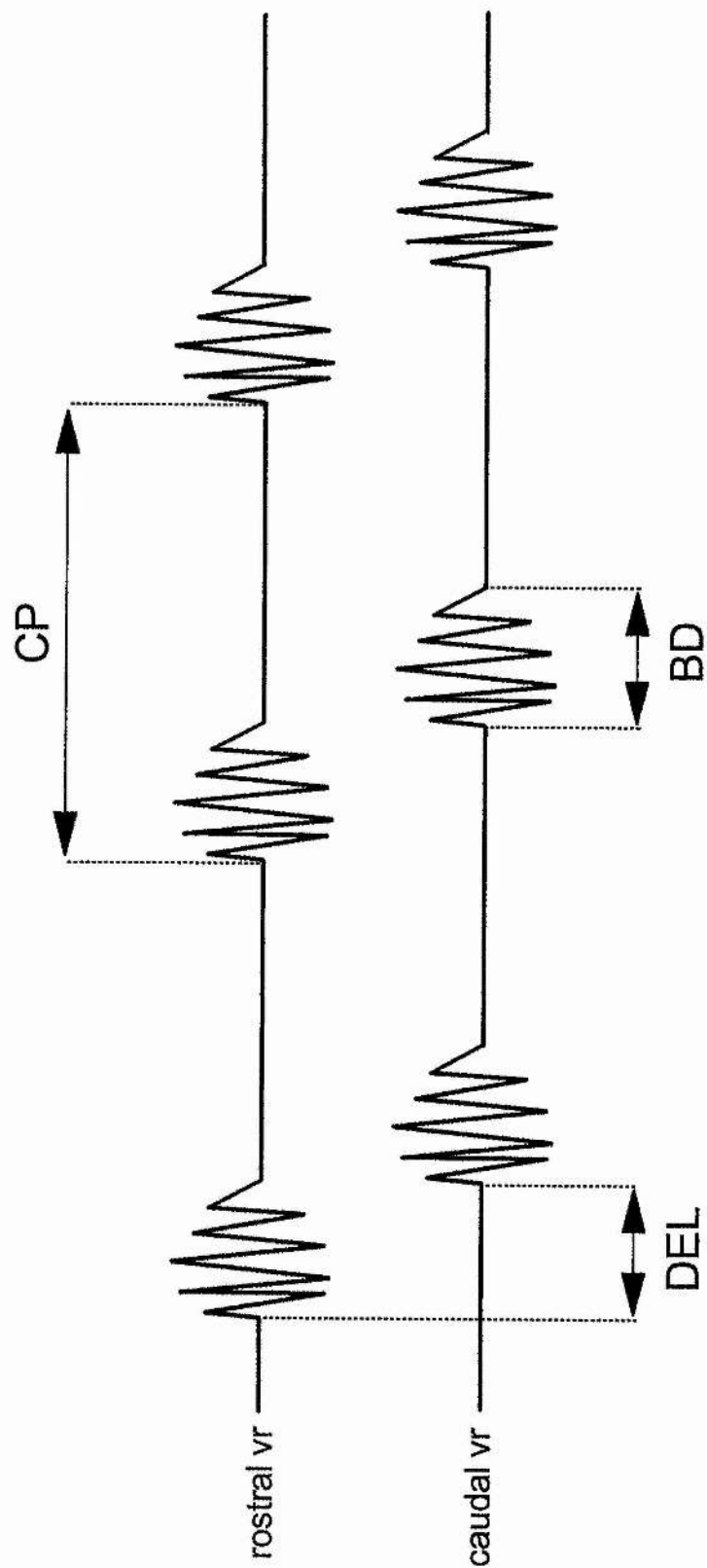
Analysis of fictive motor output

To determine the effects of drugs on ventral root activity during fictive swimming, three basic parameters were measured under each specified experimental condition. These parameter are illustrated diagrammatically in figure 2.2:

- i. Burst durations - measured (in ms) as the duration of a discrete burst of ventral root output.

Figure 2.2. Measured parameters of fictive swimming.

During fictive swimming episodes, three parameters are measured. Cycle period (CP) is measured (in ms) as the duration between the onset of one burst of ventral root output and the start of the next on the same side of the body. Burst duration (BD) is measured (in ms) as the duration of a single burst of ventral root discharge. Rostrocaudal delay (DEL) is measured (in ms) as the delay between the onset of a ventral root burst in a more rostral ventral root cleft (rostral vr) and the onset of a consecutive ventral root spike in a more caudal position (caudal vr) on the same side.



- ii. Cycle period - measured (in ms) as the time taken between the onset of one burst of ventral root activity and start of the next on the same side.
- iii. Rostrocaudal delay - measured as the delay between the onset of a ventral root burst rostrally and the onset of a consecutive ventral root burst at some more caudal position on the same side.

In order to circumvent a contribution of sensory stimulus evoked potentials to the initial cycles of swimming activity, the first 500msec of swimming activity was excluded from analysis. Unless otherwise stated in the figure legends, samples of fictive swimming used for analysis comprised thirty cycles taken from 500msec after the onset of three different episodes under each experimental condition. The mean and standard deviation for each parameter of swimming was taken from this pooled data. Although the data may not be parametric, comparisons between each condition were made using two-way t-tests, performed on Minitab for Windows (version 10). This test was used for analysing parameters of swimming because standard deviations were found to be relatively small, indicating that a parametric test could be used.

In experiments where rostrocaudal delays were measured against cycle periods, regression analysis was performed using Minitab for Windows (version 10) to establish the correlation between these two parameters of swimming. Under such circumstances, significance was tested using ANOVA.

Analysis of ipsp's

In experiments where intracellular recordings were made, the amplitude of the mid-cycle ipsp's during swimming was often measured. As phasic activity is superimposed on a level of sustained tonic depolarisation during swimming, the amplitude of the mid-cycle ipsp's was measured from the peak of the tonic drive (i.e. the base of the ipsp firing off the tonic depolarisation) to the peak depolarisation of the ipsp. Time to half-fall values for the ipsp's were calculated as

the duration (in ms) required for the ipsp to reach half maximal amplitude. Statistical comparisons between the mean ipsp amplitudes and half-fall durations were performed using the Mann-Whitney test on Minitab for Windows version 10.

CHAPTER 3

Noradrenergic modulation of the swimming motor pattern in *Xenopus laevis* tadpoles

INTRODUCTION

i) Background.

Noradrenaline (NA) is a catecholamine that has long been known to be widely distributed in the tissues of vertebrates. Today, much of our understanding of how nerve cells function has arisen directly from research on neurons that use NA as a neurotransmitter. In fact, NA was the first substance to be shown to mediate the effects of nerve cells following Langley's (1901) discovery that electrical stimulation of sympathetic nerves had a very similar effect to that which had been seen when Oliver and Schäfer (1894, 1895) intravascularly administered extracts taken from the adrenal medulla. At that time, the substance involved in producing these effects was unidentified, so when Takemine (1901) isolated the transmitter adrenaline (AD) from adrenal medulla extracts, it was mistakenly assumed that AD was the mediator of the sympathetic nerve effects seen by Langley (Elliot, 1904). It was not until the 1930s, when several differences were noted between the effects of sympathetic nerve stimulation and the action of AD, that the proposal was put forward that NA, rather than AD, was being liberated from sympathetic nerves in Langley's experiments (Bacq, 1934; Stehle & Ellsworth, 1937). Since this pioneering research, the role of noradrenergic neurons in the peripheral control of smooth and skeletal muscle contraction, heart rate, liver function, lipolysis, platelet aggregation, mast cell function and salivary gland output have all been extensively researched. However, NA containing neurons are not restricted to the PNS. It was Vogt, in 1954, who first demonstrated biochemically that NA was also found within the CNS. Later, with the advent of fluorescent staining techniques (first developed by Falk & Hillarp, 1962), insight into the full distribution of noradrenergic neurons within the

mammalian CNS was afforded (Dahlström & Fuxe, 1964). The central location of noradrenergic neurons has now been well characterised in a variety of vertebrate nervous systems. In the rat, where the majority of NA mapping has been performed, NA containing cell bodies appear to be restricted to discrete clusters (named A₁-A₇) within the pons and medulla, the axons of which branch out to send widely extending processes to many CNS regions. Of these the locus coeruleus (A₆) is the primary noradrenergic cluster comprising of 43% of the rats central NA containing neurons. Originating in the pons, the locus coeruleus forms a wide and diffuse array of arborizations from its relatively few axons (only 1,500 in the rat) which innervate the cortex, hippocampus, cerebellum and spinal cord. With respect to other vertebrates, central noradrenergic pathways have been reported in reptiles (Smeets & Steinbusch, 1989; 1990), teleosts (Ekstrom, Reschke, Steinbusch & van Veen, 1986; Hornby & Piekut, 1988; Sas, Maler & Tinner, 1990), cartilaginous fishes (Cruce, Stuesse & Northcutt, 1992) and amphibians (Gonzalez & Smeets, 1993; Gonzalez & Smeets, 1995) suggesting that they are phylogenetically ancient.

Although there have been very few attempts to map central NA pathways in the amphibian *Xenopus laevis*, it is thought that the adult *Xenopus* brain and spinal cord possess NA-containing neurons (Gonzalez & Smeets, 1993). Antibodies raised to NA have recently shown that groups of noradrenergic cell bodies are located in three primary CNS regions; i) the nucleus of the periventricular organ, extending from the dorsal hypothalamus to the lateral infundibular recess (whose CSF contacting processes are immunopositive for NA but not for tyrosine hydroxylase or dopamine- β -hydroxylase suggesting that they accumulate rather than synthesise NA); ii) the caudal brainstem (whose cell bodies extend along the ventral and medial aspects of the solitary tract) and; iii) the isthmus region (whose cells extend from just medial and ventral to the isthmus nucleus to the trigeminal nuclear complex). The spatial location of noradrenergic

cell bodies in *Xenopus* largely resemble that of mammals (Gonzalez & Smeets, 1993) and latter of these cell clusters, the isthmic region, is thought to be the amphibian homologue to the mammalian locus coeruleus. Research into the function of vertebrate central noradrenergic pathways has suggested that NA plays roles in reward systems, mood, behavioural arousal states, central blood pressure regulation and locomotion.

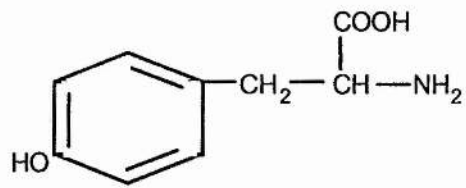
ii) NA synthesis, reuptake and metabolism.

NA shares its biosynthetic pathway with the two other catecholamines dopamine (DA) and AD. The catecholamine biosynthetic pathway is illustrated in figure 3.1. The precursor for all three of these transmitters is L-tyrosine, which is taken up by active transport into catecholamine containing cells. Once inside the cell, tyrosine is then converted to DOPA by tyrosine hydroxylase (TH). This step is the rate-limiting step of the pathway. TH is found exclusively in catecholamine containing neurones and, unlike the other enzymes involved in NA biosynthesis, it is relatively substrate-specific. DOPA is subsequently converted to DA by DOPA decarboxylase, a relatively non-specific enzyme that is also involved in catalysis of several L-aromatic amino acids such as L-histidine (the precursor to histamine) and L-tryptophan (the precursor to 5-HT). As such, this enzyme is not exclusive to catecholamine synthesising cells. DA is taken up into vesicles where it can either be stored for release or converted to NA by dopamine- β -hydroxylase, another non-specific enzyme, but one which is only found in the vesicles of catecholamine synthesising cells. NA is then either stored for transmission or converted to AD by the enzyme phenyl-N-methyl-transferase (PNMT).

Following exocytosis, NA activity is terminated by reuptake into the nerve terminal. Two main transport mechanisms exist to deal with the reuptake of NA;

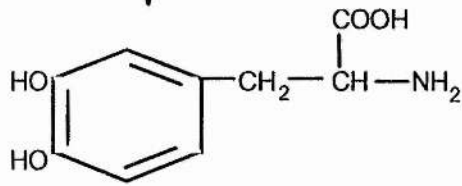
Figure 3.1. Synthesis of catecholamines from tyrosine.

Tyrosine is the precursor for the three catecholamines dopamine, noradrenaline and adrenaline.



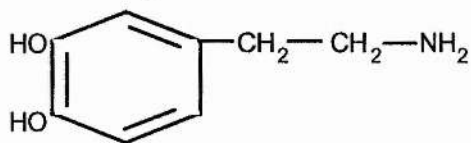
Tyrosine

Tyrosine hydroxylase



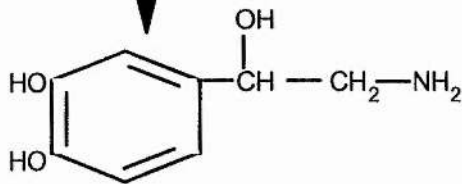
DOPA

DOPA decarboxylase



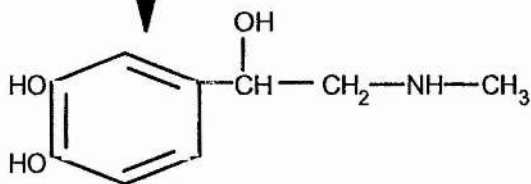
Dopamine

Dopamine β -hydroxylase



Noradrenaline

Phenylethanolamine
N-methyl transferase



Adrenaline

uptake 1 and uptake 2. Uptake 1 is neuronal with high affinity for the amine but a low rate of uptake whereas uptake 2 is an extraneuronal transporter with a low NA affinity but a high rate of uptake. Once taken back up into the nerve, a small amount of NA is repackaged directly into vesicles ready for exocytosis while the majority is broken down by two classes of metabolic enzyme; monoamine oxidase (MAO) and catechol-O-methyl-transferase (COMT). MAO, which is located on the outer membranes of mitochondria, converts catecholamines to their corresponding aldehydes via oxidative deamination. Two main forms of MAO exist, MAO-A and MAO-B. NA is primarily metabolised by MAO-A, although MAO-B will also metabolise NA if brain concentrations of the amine are sufficiently high. COMT is responsible for *O*-methylation of catecholamines resulting in the production of methoxy-derivatives. COMT is widely distributed in both neuronal and non-neuronal cells and is largely cytosolic (although a small proportion is membrane bound). Although MAO is thought to be the primary enzyme involved in NA metabolism in the CNS, both MAO and COMT can break down NA via a variety of different pathways which, following further metabolism by aldehyde reductase and aldehyde dehydrogenase, result in the production of several excretable alcohol derivatives.

iii) Adrenoceptors of the Central Nervous System.

In 1948, Ahlquist first demonstrated the existence of different adrenoceptor types by examining the potency and effects of amines on certain tissues. He suggested that the chemicals released from sympathetic nerve terminals acted on two distinct adrenoceptor classes which he termed α and β , proposing that these receptors had different affinities for NA and AD. This receptor nomenclature has persisted although it is now thought that several sub-

classes of receptor exist within each of the α and β families. In the CNS, four types of adrenergic receptor have now been identified which are distinguishable by their pharmacology, physiological action, and coupling to second messenger systems. These are termed α_1 , α_2 , β_1 , and β_2 adrenoceptors. Within each class of α adrenoceptor, several subtypes have now been cloned; the α_1 family currently consists of α_{1A} , α_{1B} and α_{1D} receptor subtypes, whilst the α_2 family, of α_{2A} , α_{2B} and α_{2C} subtypes. All adrenergic receptors found to date are metabotropic; α_1 subtypes are positively coupled to the phosphatidylinositol system, whilst α_2 subtypes are negatively, and all β subtypes positively, linked to adenylate cyclase. Although all adrenoceptor sub-types appear to mediate similar physiological effects, the central distribution of subtypes within each family is highly segregated. This regionalisation suggests differential physiological roles for each subtype of receptor in the CNS.

iv) The distribution and function of spinal NA neurones.

In the rat (where the majority of noradrenergic mapping has been performed), fibres containing NA have been shown to innervate the spinal cord in two main regions, these being the dorsal and ventral horns (Carlsson, Dahlström, Fuxe, & Hillarp, 1964; Fuxe, 1965; Clark & Proudfit, 1993; Westlund, Bowker, Ziegler & Coulter, 1983; Rajaofetra, Poulat, Marlier, Geffard & Privat; 1992; Tanaka, Takahashi, Miyamoto, Oki, Cho & Okuno, 1996). NA fibre varicosities are located in the superficial layers of the dorsal horn whilst in the ventral horn they are found around the central canal and also encircling motoneurone cell bodies. The noradrenergic origin of all spinal cord innervation appears to be the pontine cell groups A_5 - A_7 .

Axons that terminate in the dorsal horn originate from cell bodies in the locus coeruleus, subcoeruleus, parabrachial and Köelliker-Fuse nuclei (Nygren & Olson, 1977; Ader, Postema. & Korf, 1979; Westlund & Coulter, 1980; Westlund, Bowker, Ziegler & Coulter, 1981, 1983; Stevens, Hodge & Apkarian, 1982) and project caudally in the lateral funiculus (Dahlström & Fuxe, 1965; Westlund & Coulter, 1980; Stevens, Hodge & Apkarian, 1982). Axons arising from cells in the locus coeruleus descend chiefly within laminae I and II and terminate in the dorsal horn and intermediate region. Those from other pontine cell groups descend in the dorsal lateral funiculus and more ventral white matter (Fritschy & Grzanna, 1990). Within the dorsal horn, the response to NA is generally inhibitory. Iontophoretic application of NA in the dorsal horn suppresses both background activity of these cells and their response to excitatory amino acids (Biscoe, Curtis & Ryall, 1966; Engberg & Ryall, 1966; Belcher, Ryall, & Schaffner, 1978; Headley, Duggan, & Greirsmith, 1978; Fleetwood-Walker, Mitchell, Hope, Molony, & Iggo, 1985; Howe & Zieglgänsberger, 1987). Such inhibitory effects may be specific for nociceptive cells (Belcher, Ryall, & Schaffner, 1978). However, there are also reports of NA having excitatory effects on dorsal horn neurones (Weight & Salmoiraghi, 1966; Howe & Zieglgänsberger, 1987). NA-induced excitation occurs in proprioceptive cells at the base of the dorsal horn (Howe & Zieglgänsberger, 1987) as well as some cells of laminae I and II that may be inhibitory interneurons (Millar & Williams, 1989). It now appears that NA can affect different cells in different ways: experiments on neurones in laminae I and II have shown that cells in this region that are excited by NA have low thresholds and generate small action potentials upon stimulation. High threshold cells in lamina I and wide dynamic-range cells in deeper laminae are inhibited by NA (Howe & Zieglgänsberger, 1987; Millar & Williams, 1989). Overall, it appears that most nociceptive cells are inhibited by NA, although a few in laminae I and II are excited. Because inhibitory interneurons that synapse onto

nociceptive cells also appear to be excited by the amine, the main action of NA on sensory transmission would appear to be inhibitory.

There is also precedence to suggest that NA can presynaptically modulate transmitter release in the dorsal horn (Jeftinija, Semba, & Randic, 1981; Carstens, Gilly, Schreiber & Zimmermann, 1987). C-fiber presynaptic terminal excitability is increased after exposure to the α_2 agonist clonidine, an effect which is reversed by α_2 antagonists (Calvillo & Ghignone, 1986). The excitability of A fibres appears unaffected by NA. It is not yet clear whether these presynaptic effects are due to direct or indirect mechanisms. NA has also been shown to prevent the release of substance P from the spinal cord, presumably via a presynaptic mechanism (Kuraishi, Hirota, Sato, Hino, Sato & Takagi, 1985; Pang & Vasco, 1986). Therefore, NA appears to play an important role in the regulation of sensory transmission in the vertebrate spinal cord.

Noradrenergic projections descending in the ventral horn have been shown to terminate around motoneurons (Dahlström & Fuxe, 1965; Steinbusch, 1981). This suggests a role for NA in spinal motor control. The site of origin of noradrenergic motoneurone-contacting fibres in the rat is widely believed to be cell bodies in the pontine nuclei of the locus coeruleus (Westlund & Coulter, 1980; Westlund, Bowker, Ziegler & Coulter, 1983; Fritschy, Lyons, Mullen, Kosofsky, Molliver, & Grzanna, 1987; Lyons, Fritschy & Grzanna, 1989; Reddy, Fung, Zhuo, & Barnes, 1989), subcoeruleus and Kölliker-Fuse nucleus (Westlund, Bowker, Ziegler & Coulter, 1983). The effects of NA release onto motoneurons in the ventral horn have not been fully elucidated, with reports of the amines action varying. Stimulation of the locus coeruleus results in facilitation of spinal reflexes in cats (Strahlendorf, Strahlendorf, Kingsley, Gintatas, & Barnes, 1980; Fung & Barnes, 1981) and rats (Hino Ono & Fukuda, 1987; Ono, Hasebe, Satoh, Nagao, Ohta, Hirobe, & Fukuda, 1991). However the direct application of NA to ventral horn motoneurons and monosynaptic reflex (MSR) pathways have

produced inconsistent results. L-DOPA was initially shown to have no effect on the MSR in the spinal cat, but instead inhibited the polysynaptic reflex potentials induced by flexor reflex afferents (Andén, Jukes, Lundberg, & Vyklicky, 1966). Later reports in both spinal cats and rats have shown an enhancement of the MSR after exposure to NA (Barker & Anderson, 1970; Grossman, Juma & Nell, 1975).

Attempts to examine the effects of NA more directly by iontophoretic application the amine onto spinal motoneurons have also produced conflicting results. In the cat, iontophoretic application of NA was shown to hyperpolarise spinal motoneurons thereby depressing membrane excitability (Engberg & Ryall, 1966; Weight & Salmoiraghi, 1967; Phillis, Tebecis & York, 1968; Engberg & Marshall, 1971; Engberg, Flatman & Kadzielawa, 1976; Marshall & Engberg, 1979). However both α and β antagonists mimicked the NA-induced hyperpolarisations, suggesting that these effects may be non-specific (Engberg, Flatman & Kadzielawa, 1976). In direct contrast to such findings, the excitability of guinea pig trigeminal motoneurons and also rat facial and spinal motoneurons is enhanced rather than depressed by NA (Barasi & Roberts, 1977; McCall & Aghajanian, 1979; White & Neuman, 1980; Katakura & Chandler, 1990). Excitatory effects of NA have been shown to be antagonised by α -adrenoceptor antagonists (Evans & Watkins, 1978; VanderMaelen & Aghajanian, 1980; Kitazawa, Saito & Ohga, 1985; Neuman, 1985; Connell, Majid & Wallis, 1989). Recent work on cat motoneurons has suggested that NA causes slow, long latency depolarisations (White, Fung & Barnes, 1991). In the frog spinal cord, NA initially causes a short-duration motoneurone hyperpolarisation which are then followed by a prolonged depolarisation (Wohlberg, Davidoff & Hackman, 1986). It is thought that the observed depolarisations caused by NA in this preparation may to be due to decreases in motoneuronal conductance to potassium ions (VanderMaelen & Aghajanian, 1982; Larkman & Kelly, 1996). The reasons why there are conflicting reports on the effects of NA on motoneurone excitability are

not known but could be due to differences between species or to differences in inter-laboratory methodology and analysis.

Such findings have not led to any consistent conclusions as to the action of NA in the ventral horn. However, recent experiments by Ono, Fukushima and Fukuda (1993) demonstrated that the NA precursor L-DOPA facilitates ventral horn neurones and that this effect can be blocked by antagonists to α_1 adrenoceptors whilst α_2 agonists inhibit the action of L-DOPA on this region of the spinal cord. Such findings led the authors to speculate that NA acts to facilitate motoneurone excitability via an action on postsynaptic α_1 receptors located within the ventral horn whereas presynaptic α_2 receptors in the locus coeruleus reduce spinal motoneurone excitability by inhibiting the release of endogenous NA. This suggestion currently awaits validation.

Less is known regarding the action of NA on the output of spinal networks that control locomotion. In the acute spinal cat, NA agonists have been shown to be capable of inducing locomotor activity (Fossberg & Grillner, 1973; Grillner & Zangger, 1979; Barbeau, Julien & Rossignol, 1987; Keihn, Hultborn & Conway, 1992). Furthermore, in these preparations, the α_2 receptor agonist clonidine increases the cycle period of locomotor output resulting in increased flexor and extensor burst durations (Barbeau, Julien & Rossignol, 1987; Barbeau & Rossignol, 1989; Barbeau & Rossignol, 1991). Preliminary work in the lamprey has also shown that NA markedly increases cycle periods during fictive swimming (McPherson & Kemnitz, 1994). The synaptic and cellular mechanisms involved in mediating these changes in locomotion are, however, not known.

The observation that NA can exert a profound effect on locomotion provided the impetus to examine the effects of NA on the neural networks that control motor output in *Xenopus laevis* tadpoles. To date, there have not been any studies on the effects of NA on motor output in this simple model system for vertebrate locomotion. However, the CNS of the adult South African clawed frog

Xenopus laevis has been shown to contain noradrenergic fibres that innervate the spinal cord (Gonzalez & Smeets, 1993). Furthermore, it has also been shown that tyrosine hydroxylase immunopositive cell fibres are present in the spinal cord of *Xenopus* at least as early as embryonic stage 38 (Gonzalez, Marin, Tunihoff & Smeets, 1994) and although it is not known which catecholamine these fibres contain, a proportion of them may be noradrenergic. Such reports coupled with the well characterised and relative simplicity of the rhythm generating networks that control swimming (see chapter 1) made this preparation ideal for studying the effects of NA on a spinal locomotor network.

Experimental analysis of the effects of NA on fictive swimming in these tadpoles was achieved by bath applying the amine (1-10 μ M) to the *Xenopus* preparation and examining its effects on burst duration, cycle period and longitudinal co-ordination. The results obtained suggested that NA has a marked effect on the frequency of locomotion and longitudinal co-ordination during swimming episodes. Effects on burst durations were less clear. To ascertain whether NA acts at the level of the spinal cord, a further series of experiments was then carried out to investigate the effects of NA in spinal preparations. The effects were similar to those on intact tadpoles suggesting that NA acts on spinal adrenergic receptors. Finally, the pharmacology of these responses was examined via bath application of various agonists and antagonists of adrenergic receptors in an attempt to clarify the receptor sub-type(s) involved in the effects of NA. The results implicate a role for α -adrenoceptors in the NA mediated modulation of fictive swimming and also suggest that endogenously released NA may affect the swimming motor pattern.

RESULTS

i) Effects of NA on the frequency of fictive swimming.

The bath application of NA (1-10 μ M) to stage 37/8 embryos caused a reversible increase in the cycle period of swimming activity (n=6). An example of this effect is illustrated in Figure 3.2A where the amine-induced slowing of the motor pattern is immediately obvious from the excerpts of ventral root activity taken at similar points in episodes of activity recorded under each experimental condition. In this case, NA significantly ($p < 0.001$) increased cycle periods from 60.8 ± 5.9 ms in control conditions to 81.8 ± 7.7 ms, 7 minutes after application of 4 μ M NA (figure 3.2B). In order to assess the degree to which NA slows the motor pattern, the mean percentage increase in cycle period after application of NA taken from 5 representative experiments was analysed. It was found that on average, cycle periods after exposure to NA were 37.1 ± 10.7 % longer than those in control.

In the stage 37/8 embryo, swimming episodes elicited by stimulation of the tail skin begin at a high frequency and gradually decline throughout the episode until the animal stops swimming (Kahn & Roberts, 1982a). Figure 3.2C plots consecutive cycles within a complete episode of swimming in both control saline and after the bath application of 6 μ M NA. The NA-induced increase in cycle period was consistent throughout the entire episode. Another effect of NA which is apparent from this figure is an increase in the duration of swimming episodes, as shown by the increased number of cycles (n=175 under NA c.f. n=86 in control) that occur after exposure to the amine. Figure 3.3 illustrates this effect histogrammatically, where it can be seen that NA increases the average length of swimming episodes in embryos.

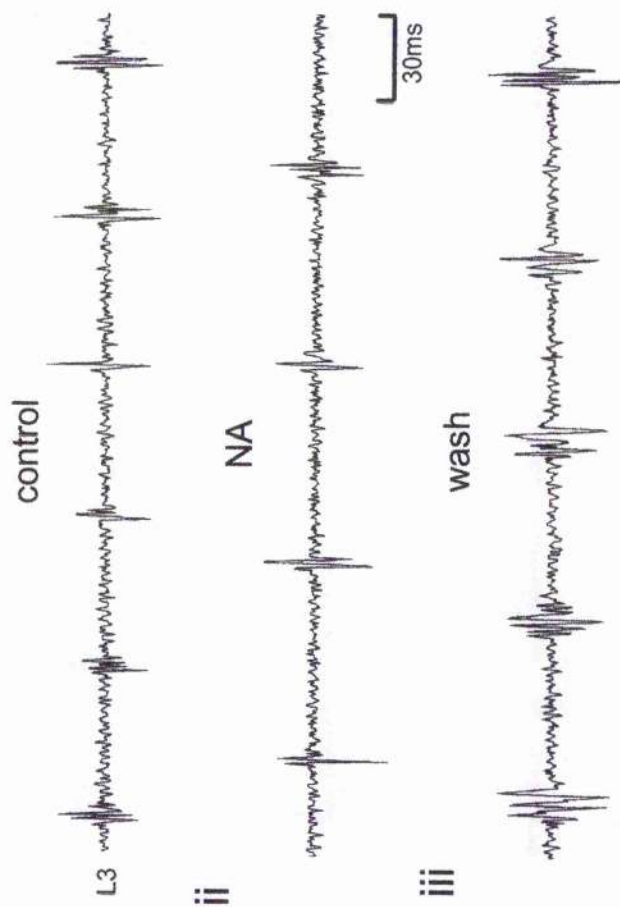
Figure 3.2. NA increases average cycle periods during embryonic fictive swimming.

A. Excerpts of ventral root activity recorded from the 3rd post otic cleft on the left side of the body (L3) and taken from equivalent points near the start of an embryo swimming episode in control (i), 7 minutes after the bath application of 4 μ M NA (ii) and after 20 minutes wash in control saline (iii).

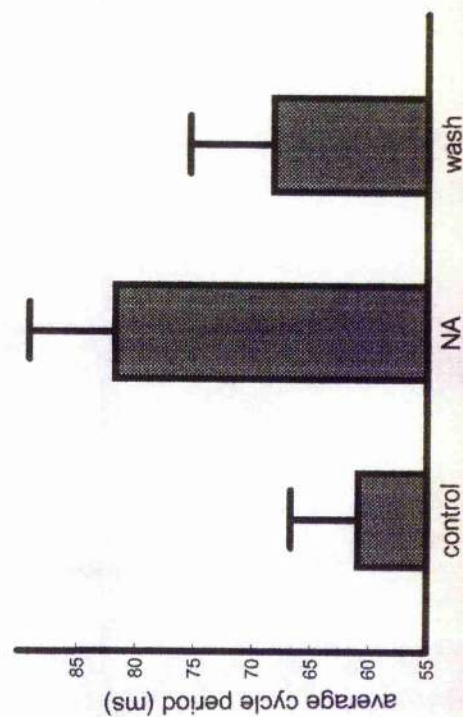
B. Histogram representing mean cycle periods under each experimental condition for the same experiment shown in A. Cycle periods increased significantly ($p < 0.001$) from 60.8 ± 5.9 ms in control to 81.8 ± 7.7 ms after exposure to NA. Subsequent washing to control saline reduced cycle periods to 68.4 ± 7.9 ms (significant, $p < 0.001$).

C. Graph depicting cycle period for each consecutive cycle of activity both in control saline (red circles, $n=86$) and 10 minutes after the bath application of 6 μ M NA (blue circles, $n=175$). It can be seen that the effects of NA are consistent throughout all cycles of activity within an episode. Data in C taken from different experiments to those shown in A and B.

Ai



B



C

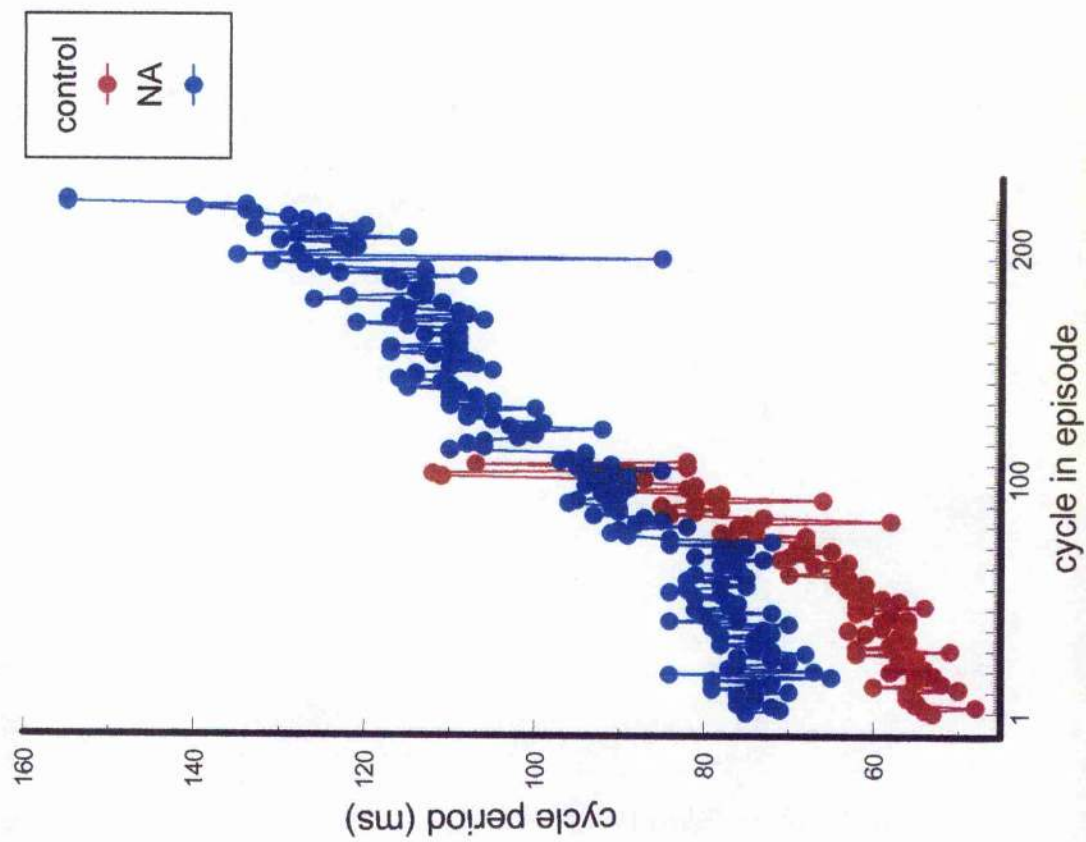
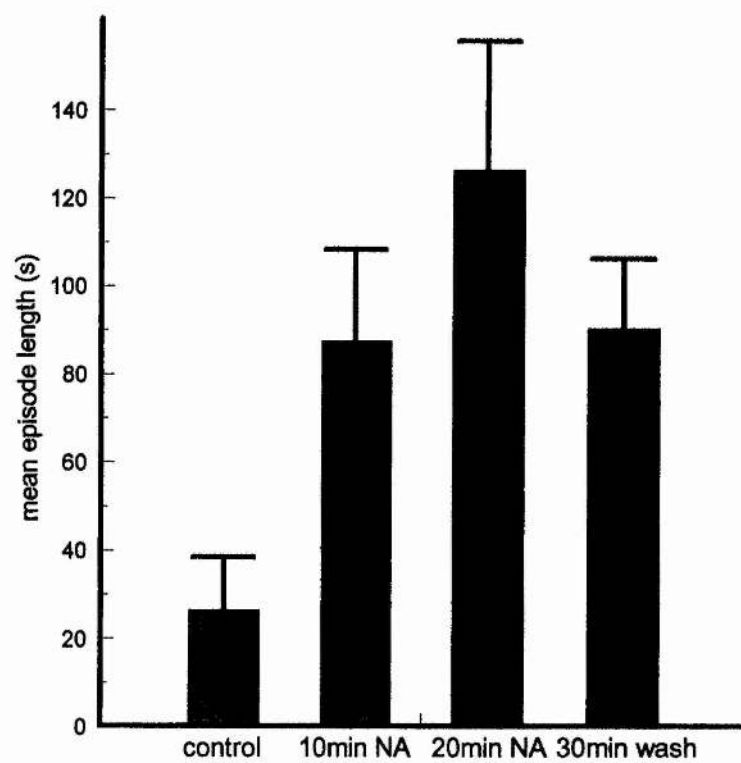


Figure 3.3. NA increases the average duration of embryonic swimming episodes.

Histogram representing the mean duration of three embryonic swimming episodes elicited in control saline, after bath application of NA and in wash. The amine causes a gradual increase in episode duration from 26.2 ± 12.9 sec in control to 86.8 ± 21.9 sec 10 minutes after the bath application of $3\mu\text{M}$ NA and 125.6 ± 28.9 sec 20 minutes after the bath application of NA. A wash for 30 minutes reduced mean episode duration to 89.4 ± 16.5 sec.



Equivalent experiments carried out on stage 42 larvae showed that bath application of NA (1-10 μ M) also reversibly increased cycle periods during fictive swimming (n=20). Figure 3.4 shows a typical example of a larval response to NA where cycle periods increased significantly ($p < 0.001$) from 51.5 ± 4.3 ms to 68.2 ± 4.8 ms after bath application of the amine (5 μ M in this case). The NA-mediated increase in larval cycle periods was similar to that seen in embryos with average cycle periods during swimming episodes taken from 5 representative experiments increasing by 34.8 ± 7.9 % after exposure to 1-10 μ M NA. Again, as for stage 37/8 embryos, the increase in cycle periods after bath application of NA in stage 42 larvae was consistent throughout all cycles within an episode (figure 3.4C). However, in contrast to the response in stage 37/8 embryos, NA markedly *reduced* larval episode lengths. This effect can be seen in figure 3.4C where, after bath application of NA, there are less cycles per episode of swimming when compared to control. The NA-mediated decrease in episode lengths is also represented histogrammatically in figure 3.5.

In both stages examined the effects of bath applied NA on motor output were reversed after around twenty to forty minutes wash in control physiological saline, so that cycle periods and episode lengths returned to around their control values. These results suggest that the *Xenopus* tadpole from as early as stage 37/8 is sensitive to NA, and that the amine is capable of producing a marked slowing of the motor pattern during bouts of fictive swimming.

ii) Effects of NA on burst durations during fictive swimming.

When NA (1-10 μ M) was bath applied to stage 37/8 embryos, no obvious effect on burst durations was observed. In stage 42 larvae, the situation was unclear with effects varying between preparations. In the majority of animals

Figure 3.4. NA increases average cycle periods during larval fictive swimming.

A. Exerpts of larval fictive swimming activity recorded from L3 and taken from equivalent points near the start of an episode in control (i), 10 minutes after the bath application of 5 μ M NA (ii) and after 30 minutes wash to control saline (iii). Note the decrease in swimming frequency under NA (ii c.f. i).

B. Histogram representing mean cycle periods under each experimental condition for the same experiment shown in A. Cycle periods increased significantly ($p < 0.001$) from 51.5 ± 4.3 ms in control to 68.2 ± 4.8 ms after exposure to NA. Subsequent washing to control saline reduced cycle periods to 52.8 ± 4.4 ms (significant $p < 0.001$).

C. Graph depicting cycle period for each consecutive cycle of activity of an entire episode of swimming activity in both in control saline (red circles, $n=288$) and 10 minutes after the bath application of 5 μ M NA (blue circles, $n=139$). It can be seen that the effects of NA are consistent throughout all cycles of activity within an episode. Data in C taken from different experiments to those shown in A and B.

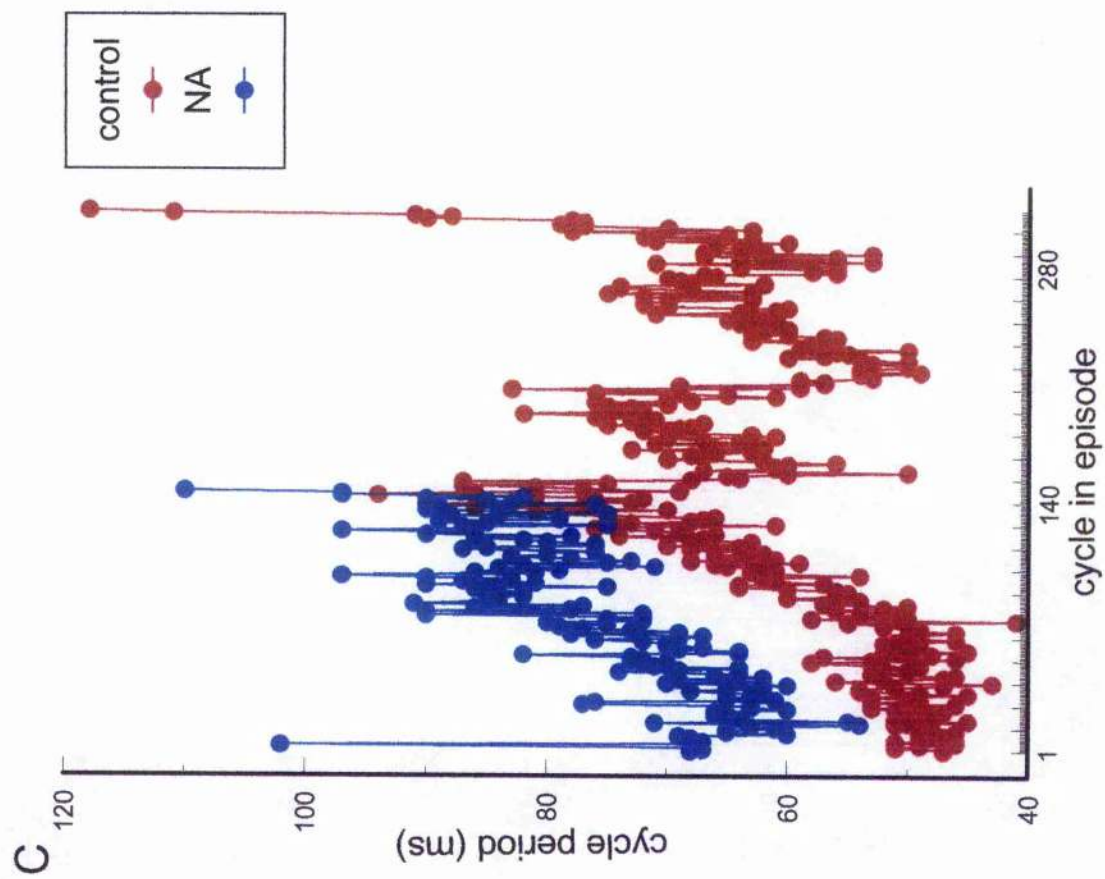
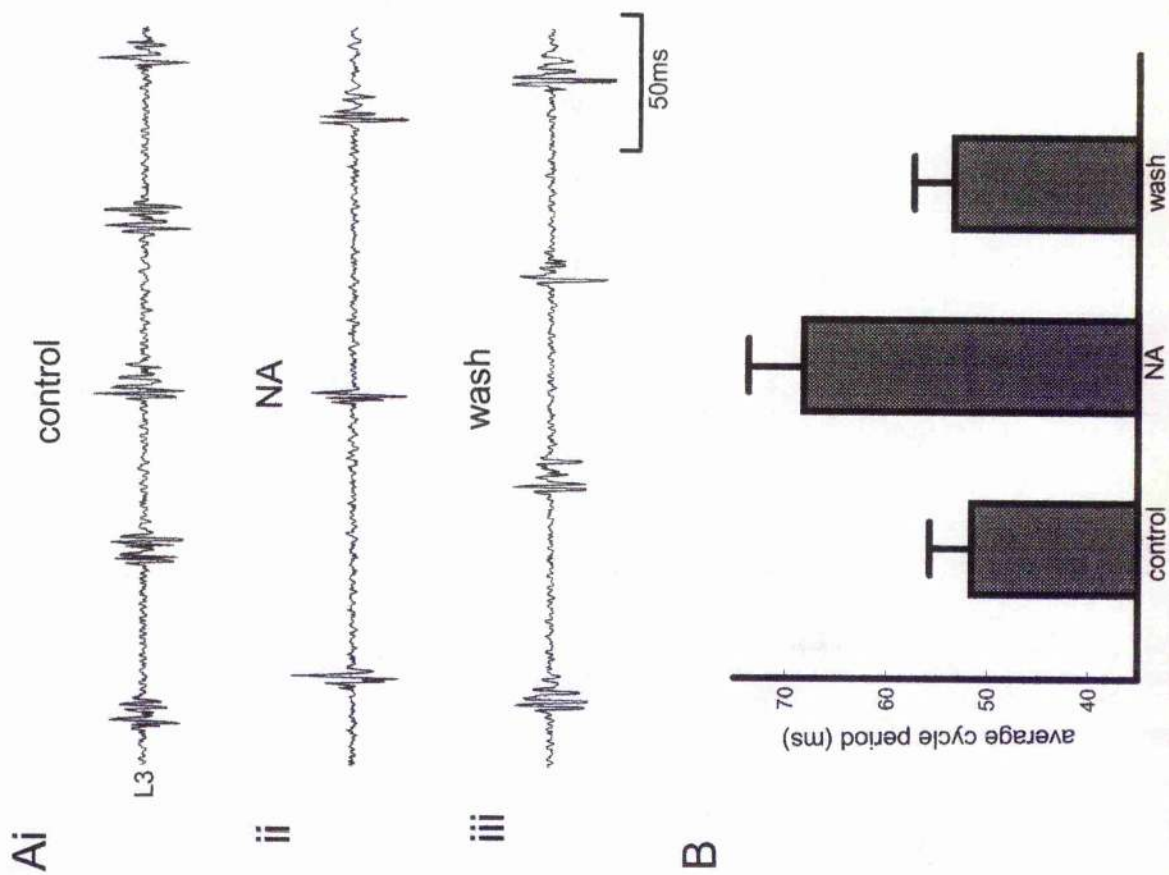
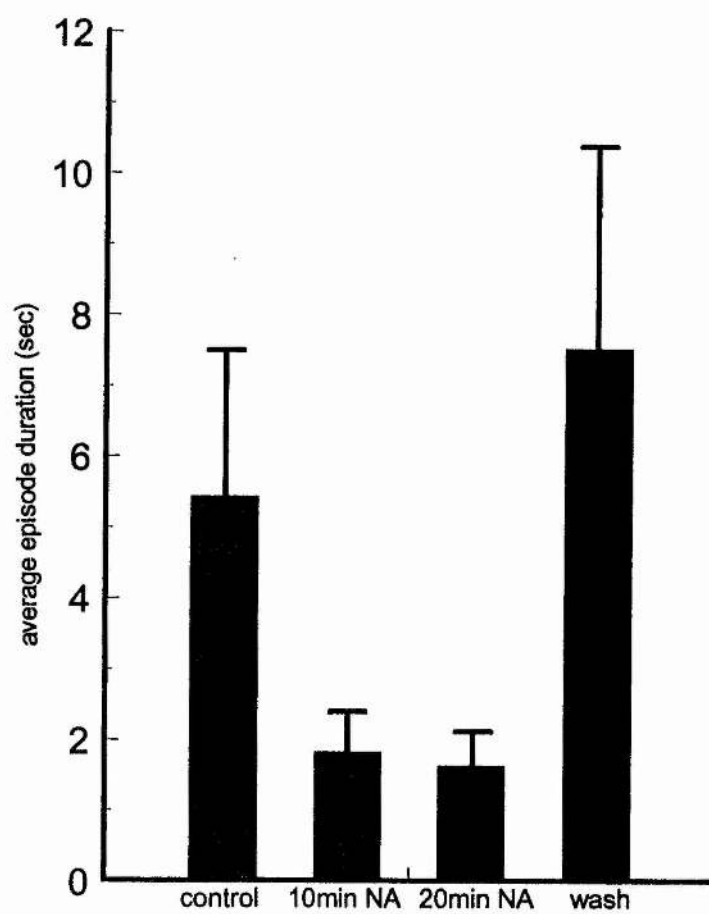


Figure 3.5. NA decreases the average duration of larval swimming episodes.

Histogram representing the mean duration of three larval swimming episodes elicited under control and after bath application of $6\mu\text{M}$ NA. A gradual decrease in episode duration is produced by the amine so that the duration of swim episodes falls from 5.4 ± 2.1 sec in control to 1.8 ± 0.6 sec 10 minutes after the bath application of NA. Mean episode duration further decreased to 1.6 ± 0.5 sec 20 minutes after the bath application of NA. A wash for 30 minutes in control saline increased episode durations to 7.5 ± 2.9 sec.



(n=9), NA did not appear to have any significant effect ($p>0.05$) on the duration of bursts during swimming episodes. However, in a small number of preparations, NA caused a significant increase (n=4) in burst durations whereas in others, a significant decrease was observed (n=2). Figure 3.6 demonstrates a typical example of NA's ability to enhance burst durations in some preparations. In this case, burst durations significantly ($p<0.001$) increased from 14.5 ± 3.7 ms in control conditions to 17.8 ± 3.9 ms after addition of NA to the recirculating saline. An example of the ability of NA to reduce burst durations is shown in figure 3.7, where mean burst durations are reduced significantly ($p<0.001$) from 13.3 ± 0.7 ms to 10.5 ± 0.8 ms after the bath application of $5\mu\text{M}$ NA. Both effects were reversible.

In some stage 42 preparations (n=11), NA could cause periods of irregular ventral root discharge which would occurred spontaneously at irregular intervals throughout episodes of fictive swimming. Figure 3.8 shows an excerpt of very regular control swimming (A) compared with swimming activity elicited 10 min after application of $5\mu\text{M}$ NA (B), during which brief periods of arrhythmic ventral root discharge could sometimes occur within the episode (arrowed in B). This effect was reversible on wash to control physiological saline (not illustrated).

iii) Effects of NA on longitudinal delays during fictive swimming.

During swimming in many animals, including the lamprey (Wallén and Williams, 1984), the leech (Pierce & Friesen, 1984, 1985), the *Rana temporaria* embryo (Soffe, 1991a) and the stage 42 *Xenopus* larva (Sillar & Wedderburn, 1993; Tunstall & Sillar, 1993) there is a positive correlation between rostrocaudal delay and cycle period so that when cycle periods increase, intersegmental delays also increase. One exception is the stage 37/8 *Xenopus* embryo. In this

Figure 3.6. Example of NA-mediated increase in burst duration.

A. Exerpts of larval swimming activity recorded from ventral root clefts L4 and L13. Exerpts are taken from near the start of an episode of fictive swimming in control (i), after bath application of 5 μ M NA (ii) and after a 20 minute wash in control saline (iii). Note the increase in burst duration after exposure to the amine (ii).

B. Histogram representing the mean burst durations under each experimental condition taken from the same experiment depicted in A. In control condition, average burst durations are 14.5 ± 3.7 ms. 10 minutes after exposure to 5 μ M NA, burst durations significantly ($p < 0.001$) increase to 17.8 ± 3.9 ms. A 20 minute wash in control saline causes the average duration of bursts to fall to 14.6 ± 3.9 ms.

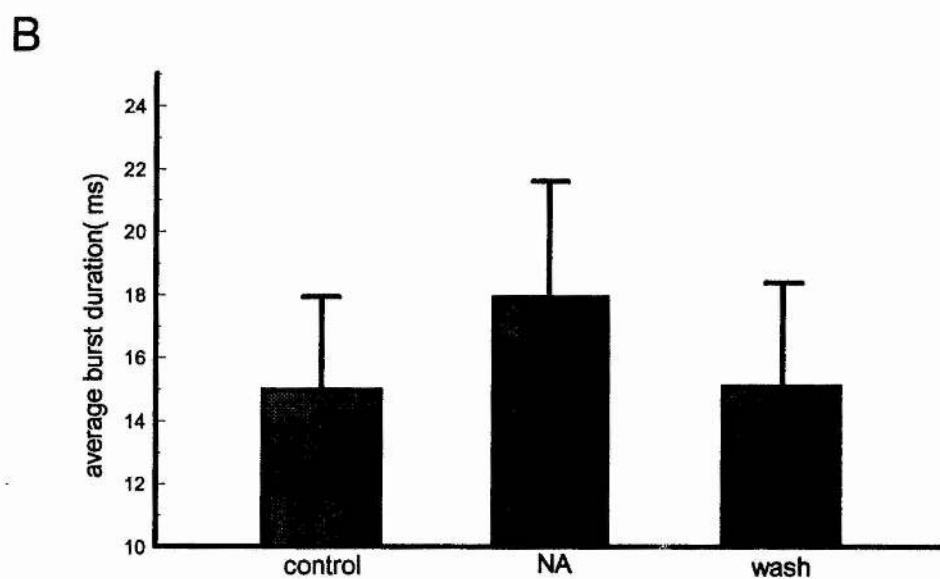
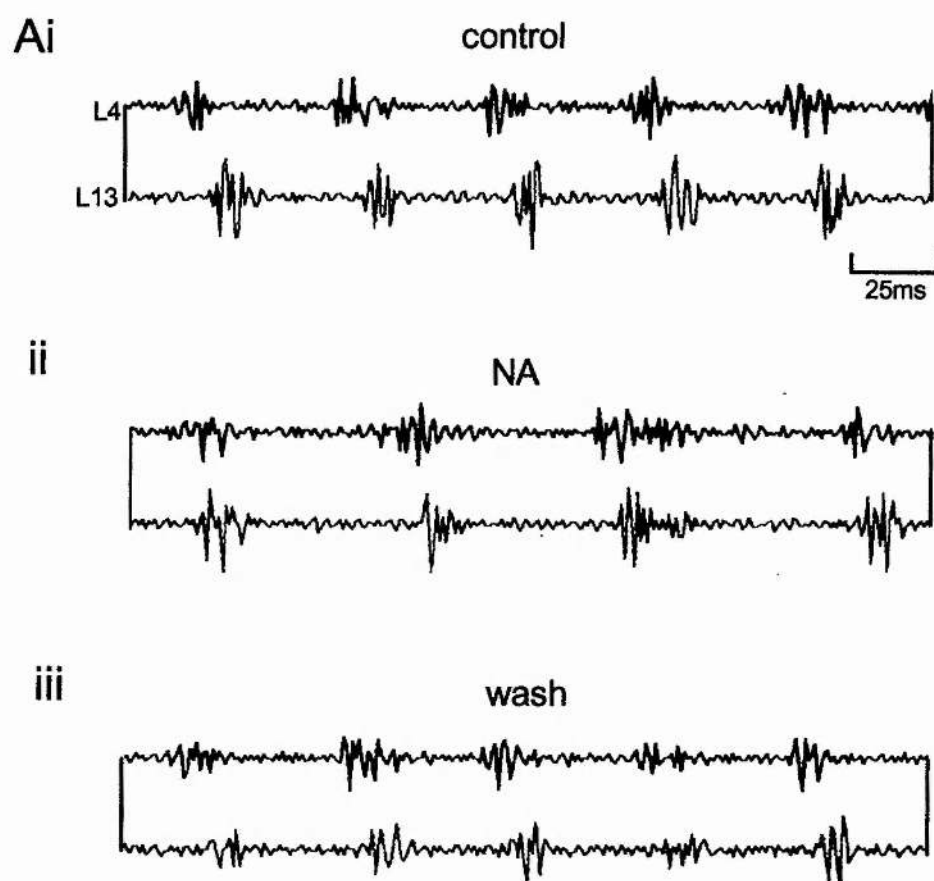


Figure 3.7. Example of NA-mediated decrease in burst duration.

A. Excerpts of larval swimming activity recorded from L3 and taken from near the start of an episode of fictive swimming in control (i), after bath application of 5 μ M NA (ii) and after a 20 minute wash in control saline (iii). Note the decrease in burst duration after exposure to the amine (ii).

B. Histogram representing the mean burst durations under each experimental condition taken from the same experiment depicted in A. In control condition, average burst durations are 13.3 ± 0.7 ms. 10 minutes after exposure to 5 μ M NA, burst durations significantly ($p < 0.001$) decrease to 10.5 ± 0.8 ms. A 20 minute wash control saline causes the average duration of bursts to increase to 12.1 ± 0.8 ms.

Ai

control



ii

NA



iii

wash



B

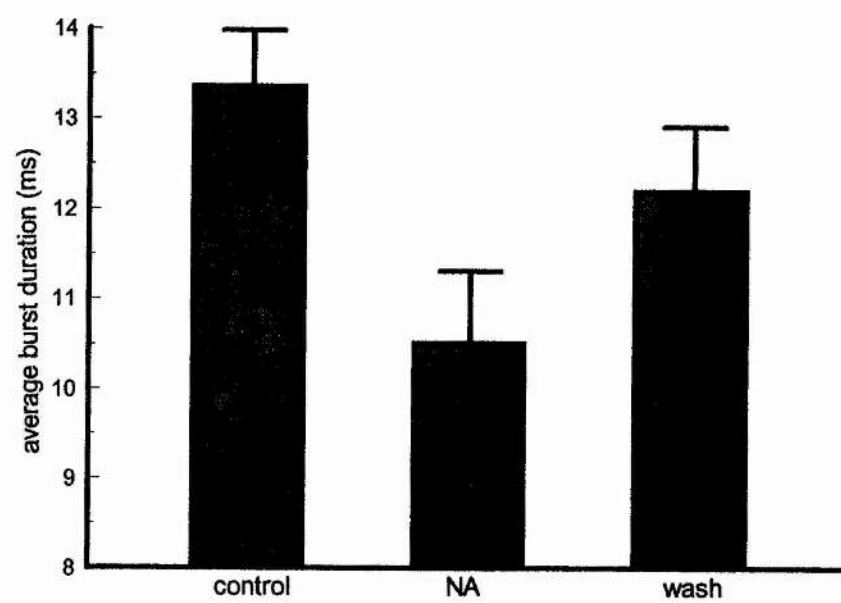
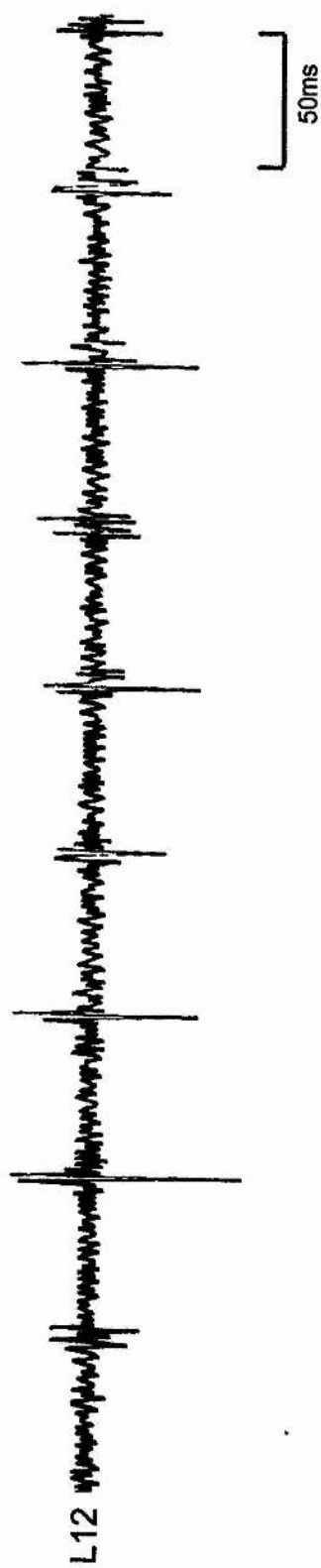


Figure 3.8. Irregular ventral root impulses produced upon exposure to NA.

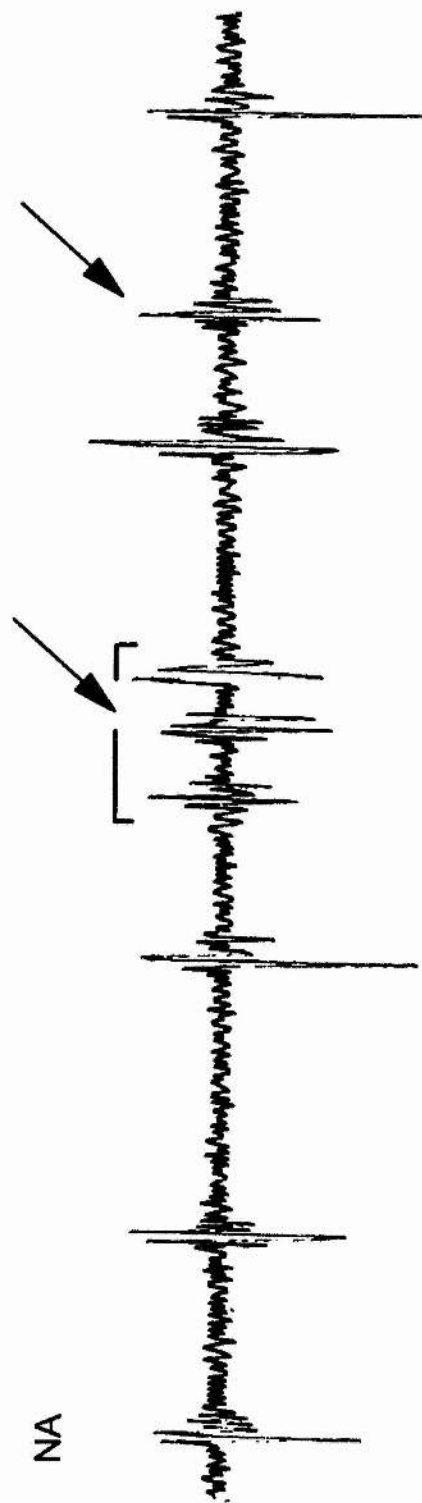
A. Excerpt fictive swimming recorded from the 12th post otic cleft (L12) and taken from around the middle of an episode of fictive swimming in a stage 42 larvae. Motor discharge is rhythmic.

B. Exerpt of fictive swimming recorded 10 minutes after exposure to 5 μ M NA. Periods of irregular ventral root discharge (arrowed) occasionally occur during episodes of swimming in the presence of the amine.

A control



B NA



preparation, there is no correlation between cycle periods and rostrocaudal delays during swimming (Tunstall & Roberts, 1991; Dale & Soffe, 1991; Tunstall & Sillar, 1993). This unusual property of the embryonic motor system is presumably due some aspects of its developmental immaturity since twenty-four hours later, at stage 42, a different scenario exists whereby there is now a positive correlation between rostrocaudal delays and cycle periods (Tunstall & Sillar, 1993). The acquisition of a constant phase-lag at this stage could be attributable to the development of serotonergic innervation to the spinal cord which occurs at around the same time in development (Van Mier, Joosten, Van Rheden & Ten Donkelaar, 1986; Sillar, Woolston & Wedderburn, 1995; Tunstall & Sillar, 1993). Indeed, physiological experiments have shown that bath application of 5-HT to stage 37/8 embryos introduces a relationship between delay and cycle period. Furthermore, in the larvae, bath application of 5-HT strengthens the relationship between these two parameters of swimming (Tunstall & Sillar, 1993). As these findings implicate a role for aminergic modulation of longitudinal co-ordination, I examined the ability of NA to modulate intersegmental co-ordination in *Xenopus* tadpoles.

The effects of NA (1-10 μ M) on longitudinal co-ordination in stage 37/8 embryos (where there is no relationship between delay a cycle period) were not looked at extensively during the course of my experiments, but preliminary data suggest that the amine causes a marked and significant reduction ($p < 0.001$) in the magnitude of the rostrocaudal delays ($n=3$). This is illustrated in the excerpt of swimming activity taken from near the start of an episode of swimming in a stage 37/8 animal (figure 3.9Ai, ii). As can be seen from the graph in figure 3.9B which plots delay against cycle period, the correlation between these two parameter of swimming is very weak ($r=0.12$), a finding that is in fitting with previous reports (Tunstall & Roberts, 1991; Tunstall & Sillar, 1993). After 10 minutes exposure to 3 μ M NA, delays decrease in duration even though swimming frequency also

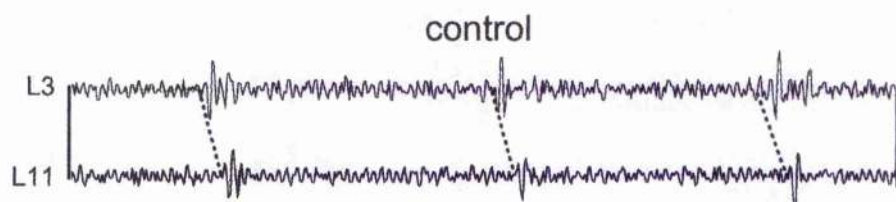
Figure 3.9. NA reduces longitudinal delays during embryonic swimming.

A. Frequency matched excerpts of stage 37/8 embryo fictive swimming activity recorded from L3 and L11 on the left side of the body in control (i), 10 minutes after the bath application of $3\mu\text{M}$ NA (ii) and after 30 minutes wash (iii). Note decrease in delay duration (depicted by dotted lines) in the presence of the amine.

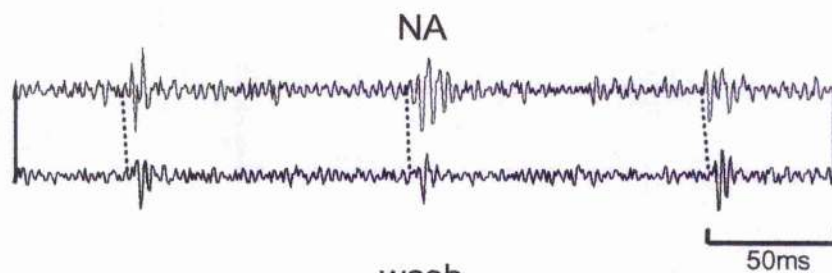
B. Graph plotting longitudinal delay against cycle period for each consecutive cycle of activity for an entire episode of swimming in control ($n=100$) and after exposure to NA ($n=113$). The correlation coefficient between these two parameters of swimming is not significant ($p>0.05$) with $r=0.12$. Following bath application of $3\mu\text{M}$ NA, there is still no significant ($p>0.05$) correlation between delay and cycle period ($r=0.21$), but delays decrease even though cycle periods increase.

C. Histogram representing the mean delays taken from three episodes of swimming under control, after exposure to NA and after wash. NA causes mean delays to significantly ($p<0.001$) decrease from 7.8 ± 2.2 ms to 4.0 ± 2.2 ms. Delays increased to 6.3 ± 2.2 ms after 30 minutes wash in control saline. Data in A, B and C taken from the same experiment.

Ai



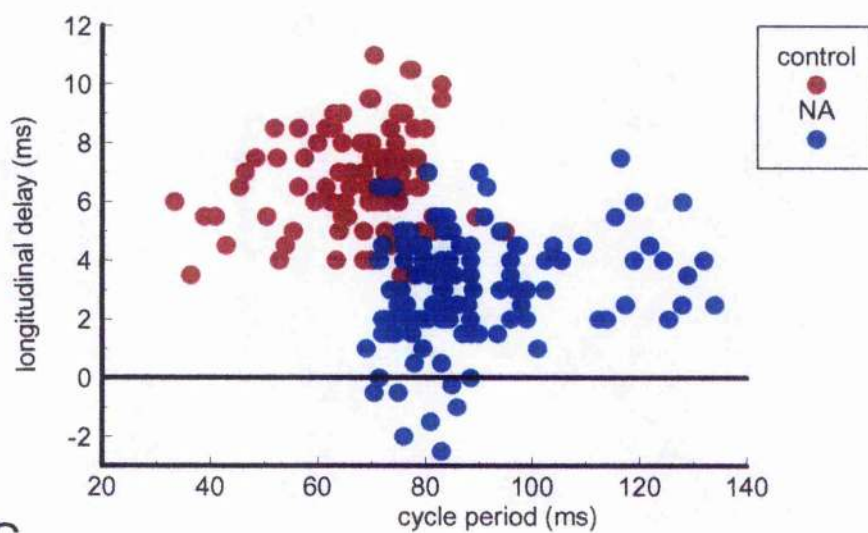
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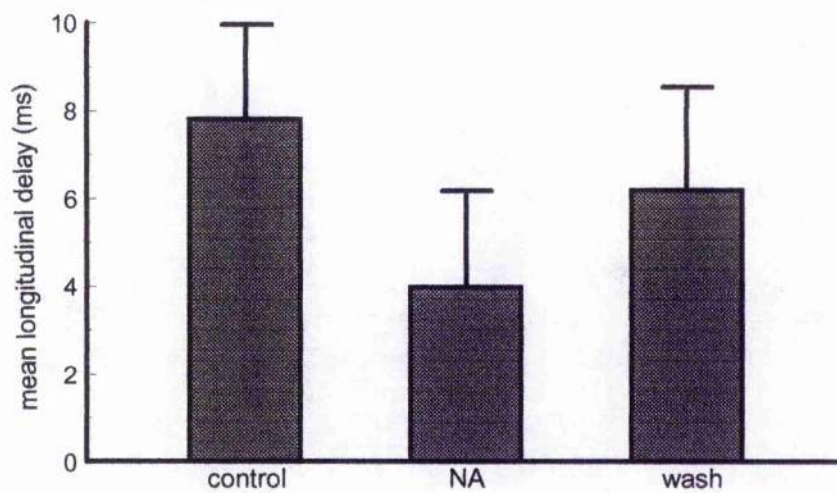
iii



B



C



decreases so that rostrocaudal delays becomes shorter for any given cycle period. The change in relationship between these two parameters is demonstrated clearly in the graph, whilst it can also be seen that the correlation between delays and cycle period remains weak ($r=0.21$) in the presence of the amine. Under NA, delays could even reverse on some cycles of activity, causing caudal ventral roots to discharge spikes before more rostral roots. The resulting effect of NA on rostrocaudal co-ordination is that, when delays are averaged throughout each entire episode, a significant ($p<0.001$) decrease in this parameter of swimming occurs compared to control activity (figure 3.9C). These effects of NA on longitudinal delays were reversible upon return to control saline (figure 3.9Aiii,C, wash).

In stage 42 larval preparations, where a relationship is known to exist between longitudinal delays and cycle periods, NA ($1-10\mu\text{M}$) also had a very distinctive effect on longitudinal co-ordination. The amine again significantly ($p<0.001$) reduced the magnitude of the rostrocaudal delays even though swimming frequency simultaneously decreases in the presence of the amine ($n=10$). An example of the effect of NA on longitudinal co-ordination in the larva is illustrated in figure 3.10 where the excerpts of activity show that delays reduce 10 minutes after bath application of $10\mu\text{M}$ NA (figure 3.10Ai, Aii). The graph in figure 3.10B plots delay against cycle period for a complete episode of swimming in both control saline and after the bath application of NA. In control conditions, the correlation between delay and cycle period is very strong ($r=0.91$), in keeping with previous findings (Tunstall & Sillar, 1993). 10 minutes after addition of $10\mu\text{M}$ NA, this correlation is maintained (although often weakened), so that delays still scale with cycle periods. However, in the presence of the amine, at any given cycle period the delay is much shorter than in control conditions. It can be seen from this graph that the effect on delays is consistent throughout an entire episode of swimming. In some preparations, NA could also reverse delays on

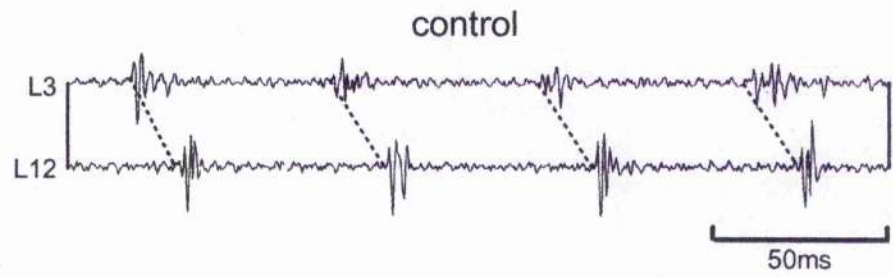
Figure 3.10. NA reduces longitudinal delays during larval swimming.

A. Frequency matched excerpts of stage 42 fictive swimming activity recorded from L3 and L12 post otic ventral root clefts in control (i), 10 minutes after the bath application of 10 μ M NA (ii) and after 30 minutes wash (iii).

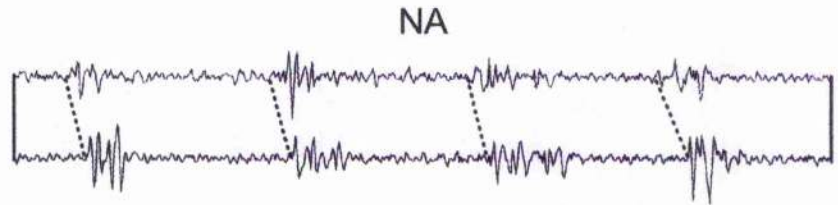
B. Graph plotting longitudinal delay against cycle period for each consecutive cycle of activity within an entire episode of swimming in control (n=214) and after exposure to NA (n=138). Data taken from the same experiment as illustrated in A. In control conditions, the correlation coefficient between delay and cycle period is highly significant ($p < 0.001$) with $r = 0.91$. Following bath application of 10 μ M NA, the correlation coefficient remains significant ($p < 0.001$) with $r = 0.83$, but the relationship between delay and cycle period shifts so that delays are shorter for any given cycle period when compared to control.

C. Histogram representing the mean delays taken from three episodes of swimming under control, after exposure to 10 μ M NA and after wash. NA causes mean delays to significantly ($p < 0.001$) decrease from 9.6 ± 2.0 ms to 5.5 ± 2.5 ms. Delays increased to 7.9 ± 2.0 ms after 30 minutes wash in control saline. Data in C is taken from a different experiment to that in A and B.

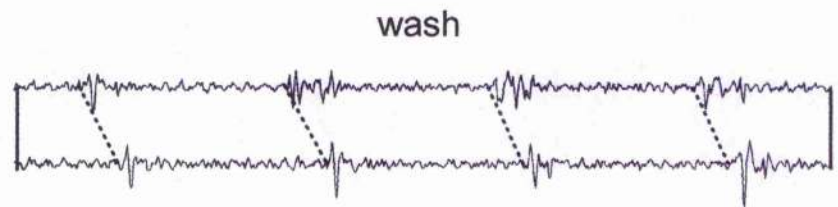
Ai



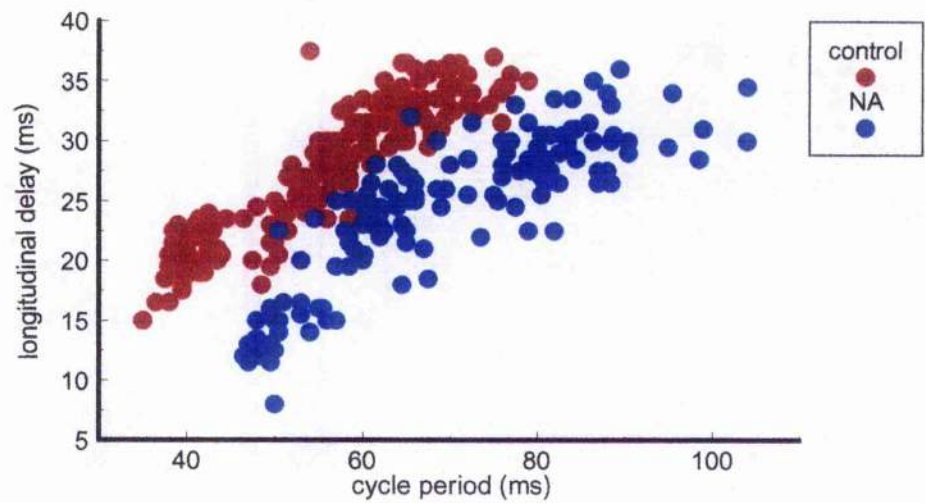
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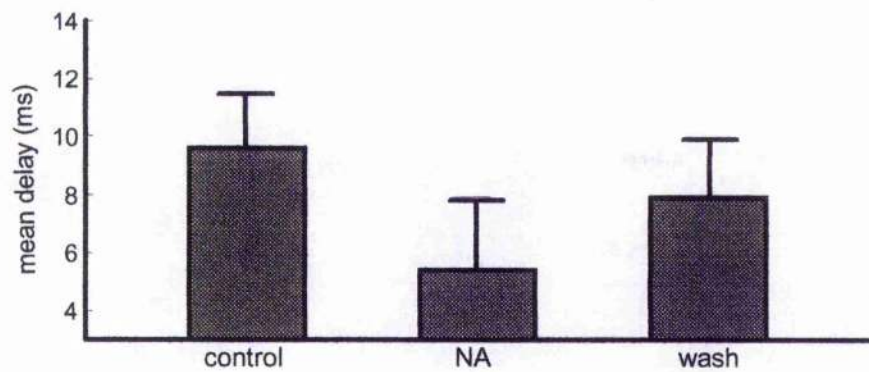
iii



B



C



some cycle of swimming so that caudal spikes discharged before more rostral ones. Thus the mean duration of the longitudinal delays during an entire episode of swimming significantly decreases in the presence of NA ($p < 0.001$, figure 3.10C).

NA therefore appears to have a profound influence on the longitudinal coordination of motor output during swimming episodes in both stages examined by markedly reducing delays at any given cycle period. Whilst NA does not appear to dramatically affect the correlation between these two parameters of swimming, it shifts them in opposite directions so that on average, delays decrease whilst cycle periods increase.

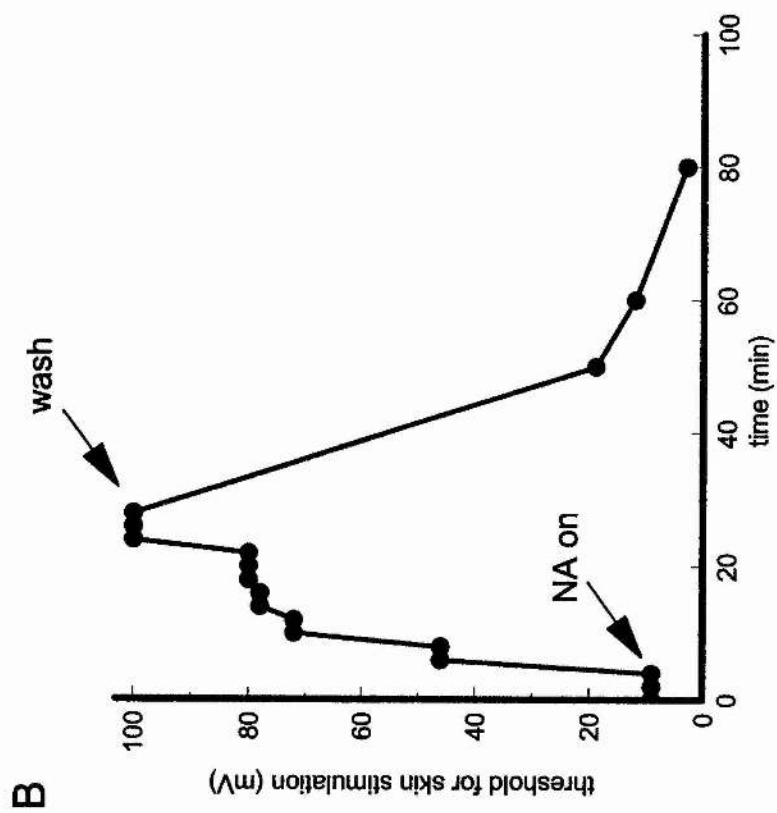
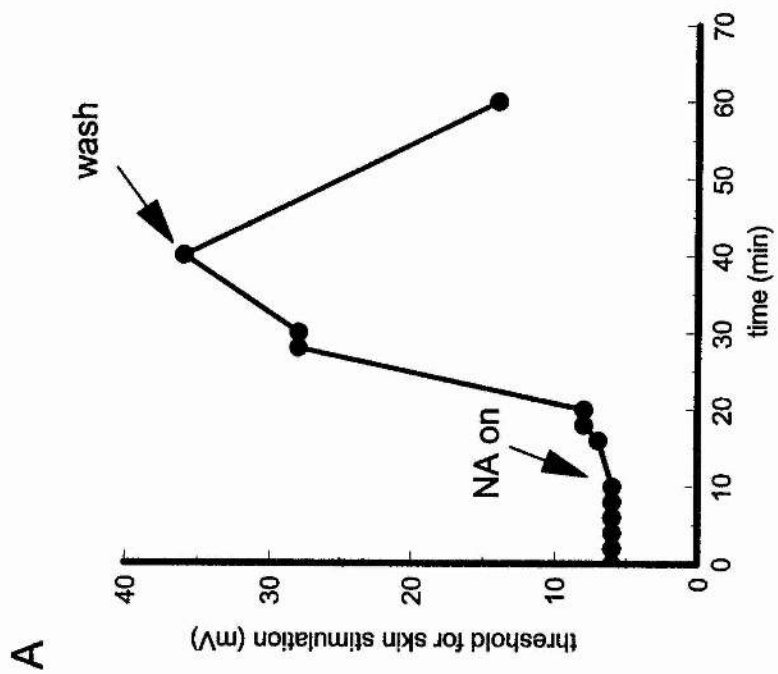
iv) Effects of NA on the threshold voltage required to initiate locomotion via the skin sensory pathway.

During the course of these experiments, it became clear that NA markedly increases the stimulus voltage required to elicit bouts of locomotor activity in response to stimulation of the skin in both embryo and larval preparations. Examples of the inhibitory effect on the threshold for skin stimulation in stage 37/8 embryos and stage 42 larvae are illustrated in figure 3.11. It is not clear whether the gradual increase in threshold seen with NA is due to the time course for the onset of the physiological effects of NA or due to a gradual build up of NA concentrations at the site of action, or a combination of the two factors.

Figure 3.11. NA increases the threshold voltage required to initiate swimming following skin stimulation in embryonic and larval preparations.

A. NA increases the minimum threshold voltage required to initiate swimming via stimulation of the tail skin in the stage 37/8 embryo. This example shows that NA ($7\mu\text{M}$ in this case) causes a time-dependent and reversible increase in the minimum voltage required to initiate rhythmic swimming activity in the embryo from 6V to 36V.

B. Example showing that NA increases threshold voltage required to initiate swimming in the larvae. The histogram shows that $3\mu\text{M}$ NA reversibly increases the minimum voltage required to initiate swimming from 9V to 100V in this case.



v) Effects of NA on transected larval swimming.

NA could act at two possible sites in the CNS to affect the final output of locomotor networks in *Xenopus* tadpoles: it could mediate changes within higher centres of the brain or the brainstem which then exert a descending influence on the spinal networks that control locomotor output; conversely, NA could act directly on receptors located on spinal neurones. In order to clarify where the receptors for NA's actions are located, experiments were performed on stage 42 larvae that had been transected at the level of the first post-otic cleft (see figure 3.12A), thereby removing influences from the brainstem and higher centres. If NA did not alter motor output in transected preparations, this would indicate that the amine acts on higher centres within the brain. Conversely, if NA was still capable of affecting motor output in transected preparations, then it is likely the amine acts directly on the spinal networks for locomotion. Transection experiments were carried out only on stage 42 preparations since at this older stage, the CPG's for locomotion are likely to be under a stronger noradrenergic influence than at stage 37/8.

Immediately after transection, larval preparations produced brief, embryonic-like bursts of ventral root discharge and swam at a much lower frequency when compared to control conditions (Sillar & Woolston, 1995). Animals were therefore allowed to recover for 20 to 30 minutes after transection before experiments were carried out. During this recovery period, animals were placed in fresh ringer, to remove any neuroactive substances that may have leached into the saline as a result of the transection procedure. As previously reported by Sillar and Woolston (1995), after this time, preparations resumed a more typically larval, bursty pattern of ventral root discharge and regained the ability to swim at a frequency more representative of that seen in intact larval animals.

In transected preparations (figure 3.12A), as illustrated by the excerpts of ventral root activity shown in figure 3.12B, NA (0.5-5 μ M) still markedly increased cycle periods (n=10, figure 3.12Bii) when compared to control transected activity (figure 3.12Bi). In this experiment the mean cycle period increased significantly ($p < 0.001$) from 64.7 ± 9.2 ms to 77.9 ± 9.8 ms after exposure to 1 μ M NA, an effect which was reversible upon return to control saline. The mean increase in cycle period taken from 5 representative experiments was found to be 33.0 ± 21.6 %, very similar to the increase seen in intact larvae (34.8 ± 7.9 %). Increases in cycle periods were consistent throughout all cycles within an episode, an effect which is illustrated in figure 3.12D.

The effects of NA on the relationship between longitudinal delays and cycle periods were also examined in transected larvae (n=4). It was found that in the absence of any influence from higher centres, the correlation between these two parameters of swimming was often weaker than that seen in intact animals (figure 3.13B). Subsequent bath application of NA (0.5-5 μ M) was found not to substantially reduce delays relative to cycle period (figure 3.13B), and indeed mean delays did not significantly ($p > 0.05$) decrease in the presence of the amine (figure 3.13C). One problem when studying relationships between delay and cycle period in these animals was that transection markedly reduced episode lengths so that very few cycles remained within an episode. Subsequent addition of NA reduced episode durations even further, so that only a few cycles often remained. Therefore, comprehensive analysis of the relation between cycle period and delay in transected animals was not possible. It is therefore presently not clear whether the amine can affect delays in transected animals.

With respect to the other parameters of swimming, NA did not have any significant effect ($p > 0.001$) on burst durations in all transected preparations tested (n=10), although there was often a small, but non-significant decrease in burst durations after exposure to the amine. NA had similar effects on both the

Figure 3.12. NA increases average cycle periods during swimming in larvae transected at the level of the first post otic cleft.

A. Stage 42 larvae were transected at the level of the first post otic cleft (approximately at arrow) to remove descending influences from higher centres.

B. Exerpts of fictive swimming activity recorded from L6 and taken from equivalent points near the start of an episode in control (i), 4.5 minutes after the bath application of $1\mu\text{M}$ NA (ii) and after 25 minutes wash in control saline (iii).

C. Histogram representing mean cycle periods under each experimental condition for the same experiment shown in B. Cycle periods increased significantly ($p < 0.001$) from 64.7 ± 9.2 ms in control to 77.9 ± 9.8 ms after exposure to NA. Subsequent washing in control saline reduced cycle periods to 56.2 ± 10.9 ms (significant to $p < 0.001$).

D. Graph depicting cycle period for each consecutive cycle of activity both in control saline (red circles) and 10 minutes after the bath application of NA (blue circles). Note that the effects of NA are consistent throughout all cycles of activity within an episode. Data taken from a different experiment to B and C.

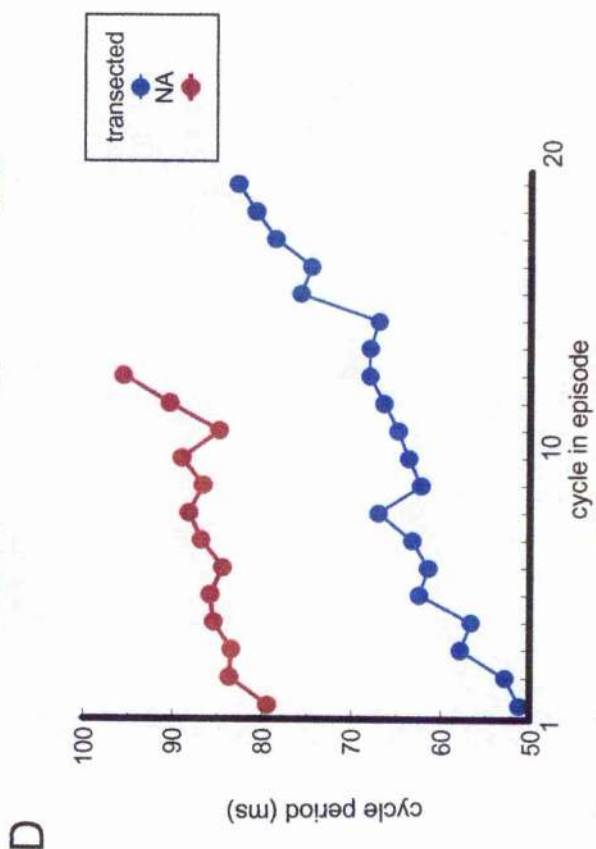
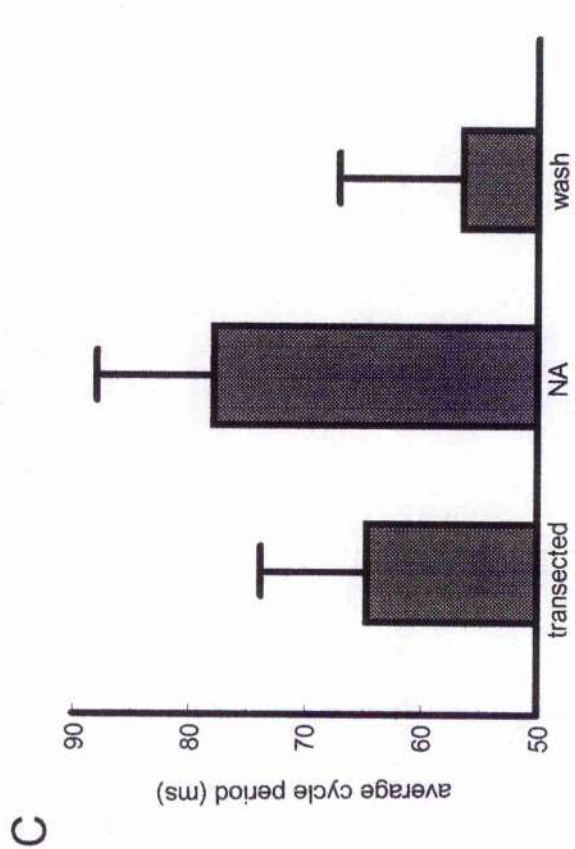
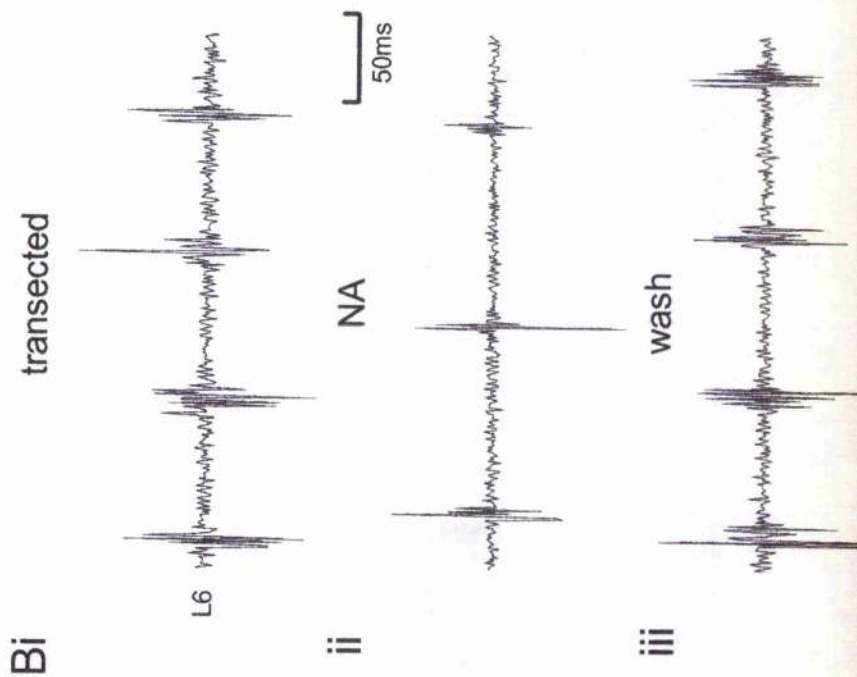
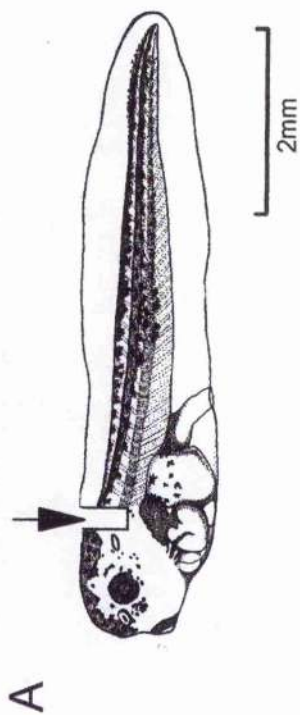


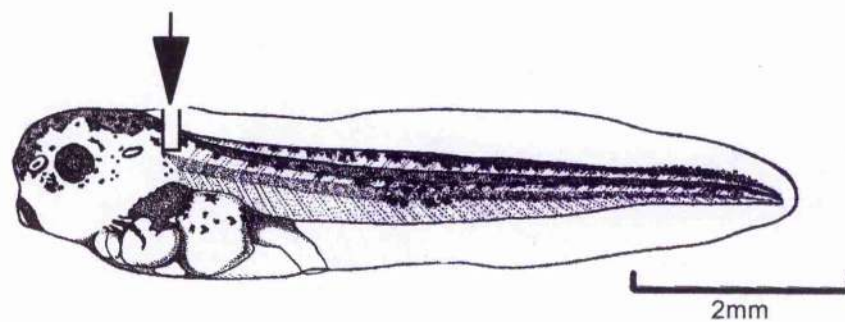
Figure 3.13. Effects of NA on longitudinal delays during swimming in larvae transected at the level of the first post otic cleft.

A. Stage 42 larvae were transected at the level of the first post otic cleft (approximately at arrow) to remove influence from higher centres.

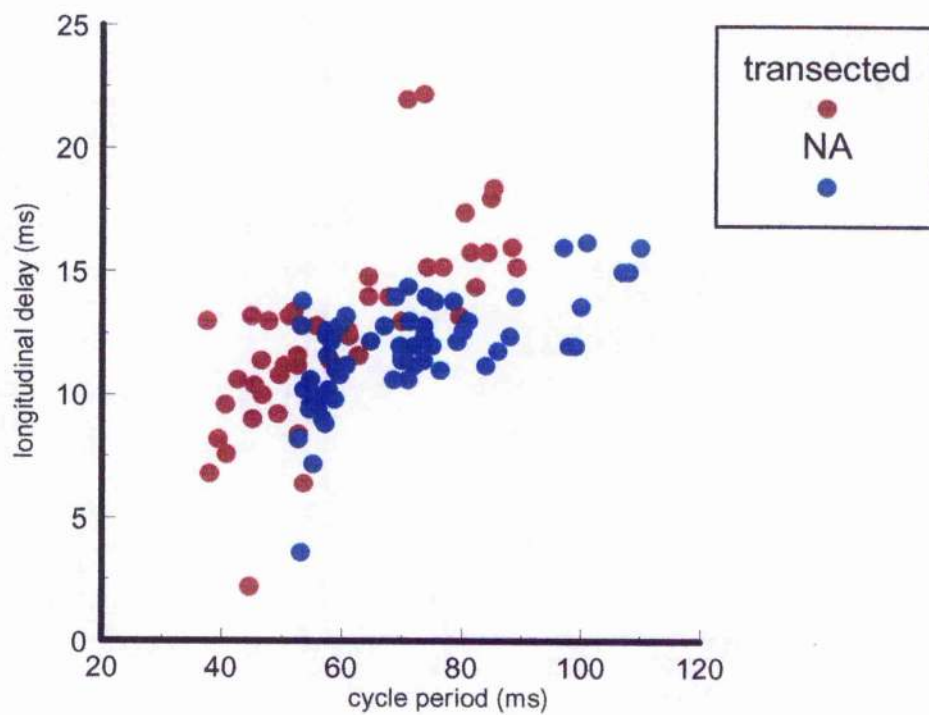
B. Graph plotting longitudinal delay against cycle period for each consecutive cycle of activity taken from three episodes of swimming in control saline ($n=47$) and from three episodes elicited after 10 minutes exposure to NA ($n=48$). The relationship between delay and cycle period is weakened in the transected preparation under control conditions ($r=0.72$). Following bath application of $1\mu\text{M}$ NA, the correlation between delay and cycle period remains weak ($r=0.53$). It can be seen that there is little shift in the relationship between delay and cycle period in the presence of NA.

C. Histogram representing the mean delays taken from three episodes of swimming under control, after exposure to NA and after wash. In control conditions, mean delays were 12.8 ± 3.2 ms. NA caused a small decrease in mean delays so that they were now 11.3 ± 2.6 ms, a change that was not significant ($p>0.05$). Delays were 11.7 ± 4.4 ms after 35 minutes wash to control saline.

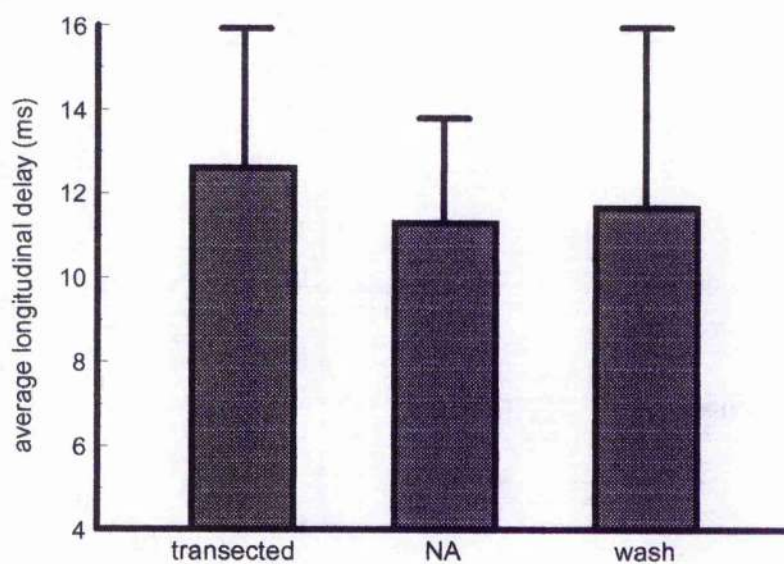
A



B



C



threshold for skin stimulation and the duration of episode lengths to those seen in intact animals. In figure 3.14A it can be seen that exposure to the amine still caused a significant decrease ($p < 0.001$) in the duration of episodes of swimming, an effect which was difficult to fully reverse in these transected preparations. The reduction in episode length was often so great that episodes elicited as little as four minutes after exposure to NA often only comprised two to three cycles of activity. NA would also increase the threshold for skin stimulation in transected larvae, a typical example of which is represented graphically in figure 3.14B. The concentration of NA required to affect the motor pattern and the time course for the onset for these effects were greatly reduced in transected animals (around a four minute incubation of 0.5-1 μ M NA usually produced marked effects on the motor pattern). Indeed, if concentrations higher than this were used, it often became impossible to elicit any rhythmic activity upon stimulation. The increased potency of NA in these preparations is likely to reflect the much enhanced accessibility of the drug into the CNS following spinal cord transection. The fact that NA can still have a strong influence on the swimming system in preparations that have been transected at the level of the first post-otic cleft indicates that NA is acting upon receptors on spinal neurones rather than in the brain or the brainstem.

vi) A possible role for α -adrenoceptors in the effects of NA.

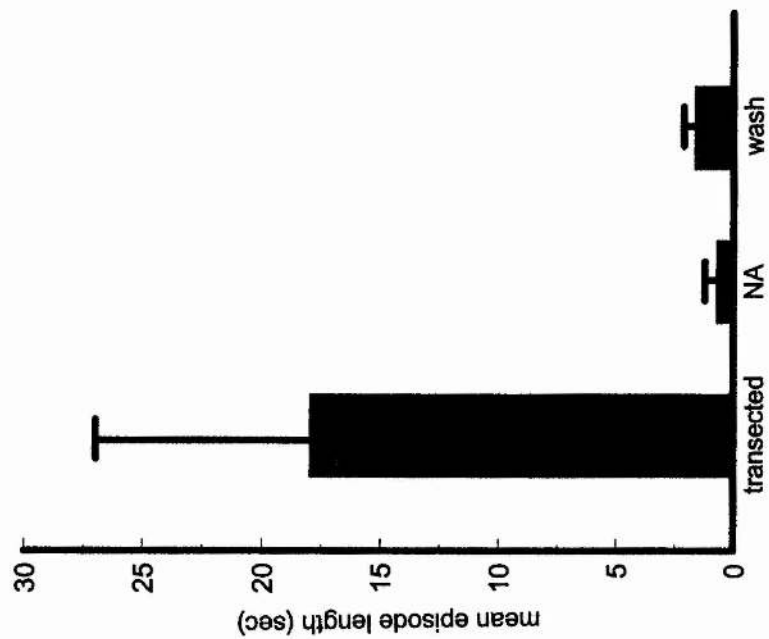
In order to investigate which adrenoceptor subtype is involved in mediating the effects of NA, a range of adrenergic agonists and antagonists were applied to preparations and their effects on the swimming motor pattern was studied. Again, as for transected preparations, pharmacological experiments were performed on stage 42 preparations because it is likely that the CPG for

Figure 3.14. Effects of NA on episode length and threshold voltage required to initiate swimming in larvae transected at the level of the first post otic cleft.

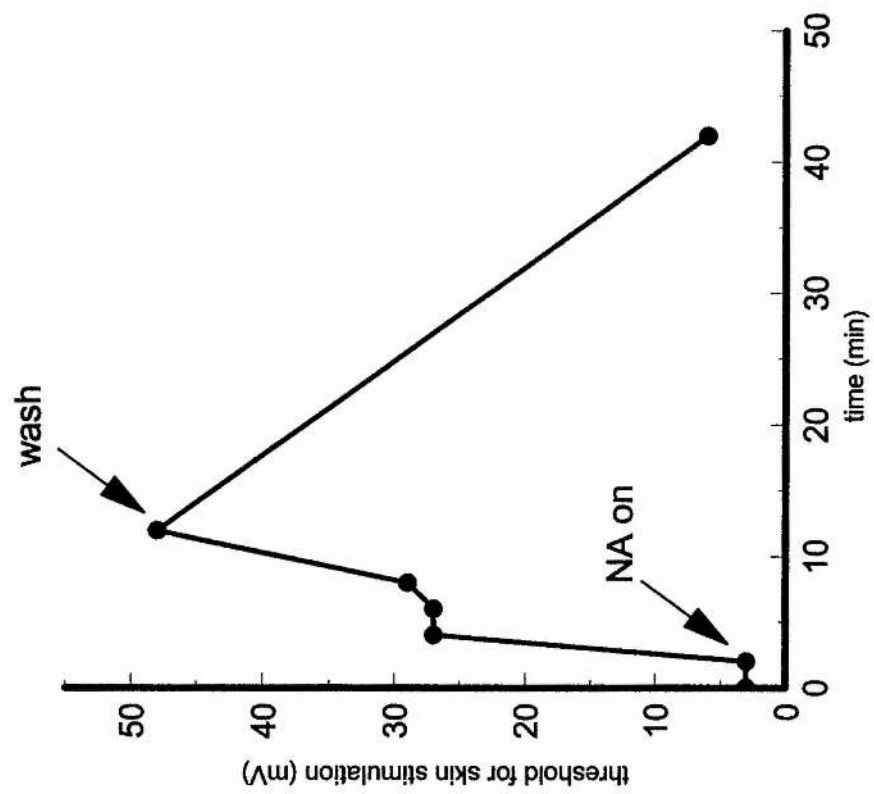
A. NA decreases the mean episode length in transected stage 42 larvae. In control conditions, episodes were, on average 17.9 ± 9.2 sec. After four minutes bath application of $1\mu\text{M}$ NA, mean episode length fell to 0.6 ± 0.5 sec. A wash for 20 minutes in control saline only slightly reversed the effects of NA, with mean episode length increasing to 1.6 ± 0.7 sec. The average episode length was calculated as the mean duration of three episodes under each experimental condition.

B. NA increased the threshold voltage required to initiate swimming activity in a transected larva from 3V to 48V, an effect that is reversible upon wash to control saline.

A



B



locomotion is under a stronger noradrenergic influence at this later stage in development. Initial experiments involved exogenous application of agonists for both α and β adrenoceptor subclasses, in order to attempt to mimic the effects of NA. However, none of these agents reproduced any of the effects seen with NA. The agonists tested were the α_1 agonist phenylephrine (5-50 μ M, n=9, figure 3.15A), the α_2 agonist clonidine (1-100 μ M, n=12, figure 3.15B), and the broad-spectrum β agonist isoprenaline (5-100 μ M, n=4, figure 3.15C). None of these compounds affected any of the parameters of fictive swimming activity such as cycle period, burst duration, rostrocaudal delay. The lack of effect of these relatively selective agents may be due to either a requirement for co-activation of more than one receptor subtype to reproduce the effects of NA or the fact that amphibian adrenergic receptors are pharmacologically distinct from their mammalian counterparts (c.f. O'Donnell & Wanstall, 1982).

The effect of the broad-spectrum α -antagonist phentolamine was therefore studied. When NA was bath applied to stage 42 larvae in the presence of phentolamine (20-50 μ M), NA could no longer elicit an effect on either cycle period (n=11) or rostrocaudal delay (n=9). A typical example of the ability of phentolamine to occlude NA's ability to increase cycle periods is shown in figure 3.16. Here it can be seen that whilst average cycle periods increased significantly ($p < 0.001$) from 54.8 ± 4.1 ms to 81.1 ± 11.5 ms under 10 μ M NA alone, the amine had no significant effect when bath applied in the presence of 50 μ M phentolamine.

Phentolamine was also capable of blocking the NA-mediated effects on longitudinal co-ordination. Figure 3.17 shows that where NA alone reduces rostrocaudal delays over a range of cycle periods, subsequent addition of phentolamine in the presence of the catecholamine causes an increase in delays and a reduction in cycle period (figure 3.17A,B). Phentolamine caused the mean longitudinal delays during swimming to increase significantly ($p < 0.001$) after

Figure 3.15. Lack of effect of noradrenergic agonists on larval fictive swimming.

A. Histograms showing lack of significant ($p > 0.05$) effect of the α_1 -agonist phenylephrine ($10\mu\text{M}$) on both burst durations (i), cycle periods (ii) and rostrocaudal delays (iii) during stage 42 larval swimming.

B. Clonidine, an α_2 -agonist ($10\mu\text{M}$) also does not significantly ($p > 0.05$) affect either burst durations (i), cycle periods (ii) or rostrocaudal delays in stage 42 larvae.

C. The broad-spectrum β -agonist isoprenaline ($10\mu\text{M}$) was equally incapable of modulating larval motor output during swimming, so that burst durations (i), cycle periods (ii) and rostrocaudal delays were not significantly ($p > 0.05$) affected in the presence of the agonist.

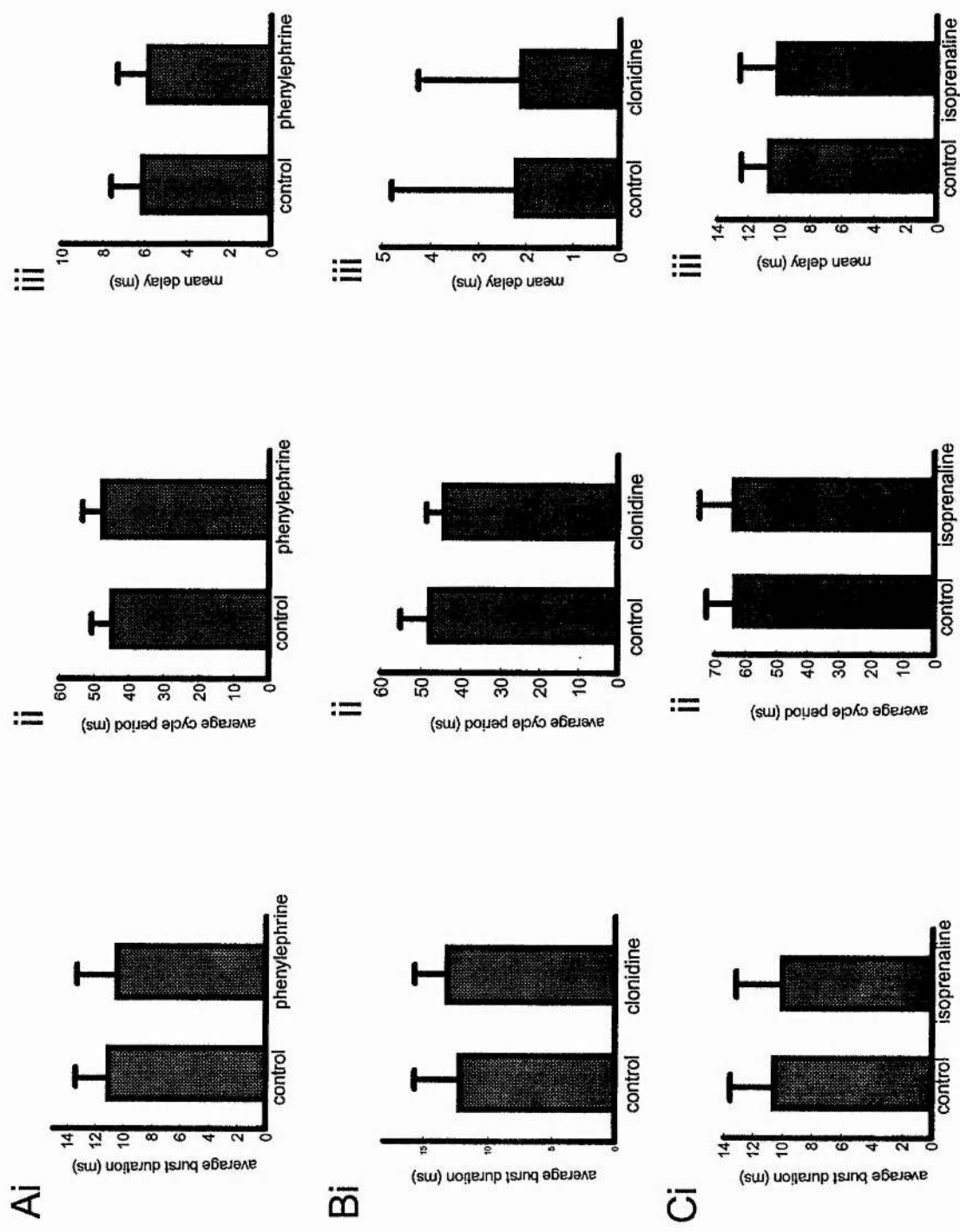


Figure 3.16. Phentolamine blocks the effects of NA on cycle period during larval swimming.

A. Exerpts of ventral root activity recorded from L6 and taken from equivalent points near the start of an episode of stage 42 larval swimming in control (i), 10 minutes after the bath application of 10 μ M NA (ii), after 20 minutes wash (iii), 20 minutes after the bath application of 50 μ M phentolamine (iv), after subsequent addition of 10 μ M NA (v) and after a 30 minute wash to control saline (vi).

B. Histogram showing that NA does not increase mean cycle periods in the presence of NA. Cycle periods significantly ($p < 0.001$) increased from 54.8 ± 4.1 ms to 81.1 ± 11.5 ms after bath application of 10 μ M NA. Wash to control saline reduced average cycle periods to 52.7 ± 4.4 ms. Average cycle periods were 52.2 ± 3.4 after 20 minutes exposure to 50 μ M phentolamine. Subsequent re-addition of 10 μ M NA did not significantly affect cycle period durations, with the mean remaining at 52.2 ± 5.0 ms. Cycle periods were 53.2 ± 4.8 ms after return to control saline. Data in A and B taken from the same experiment.

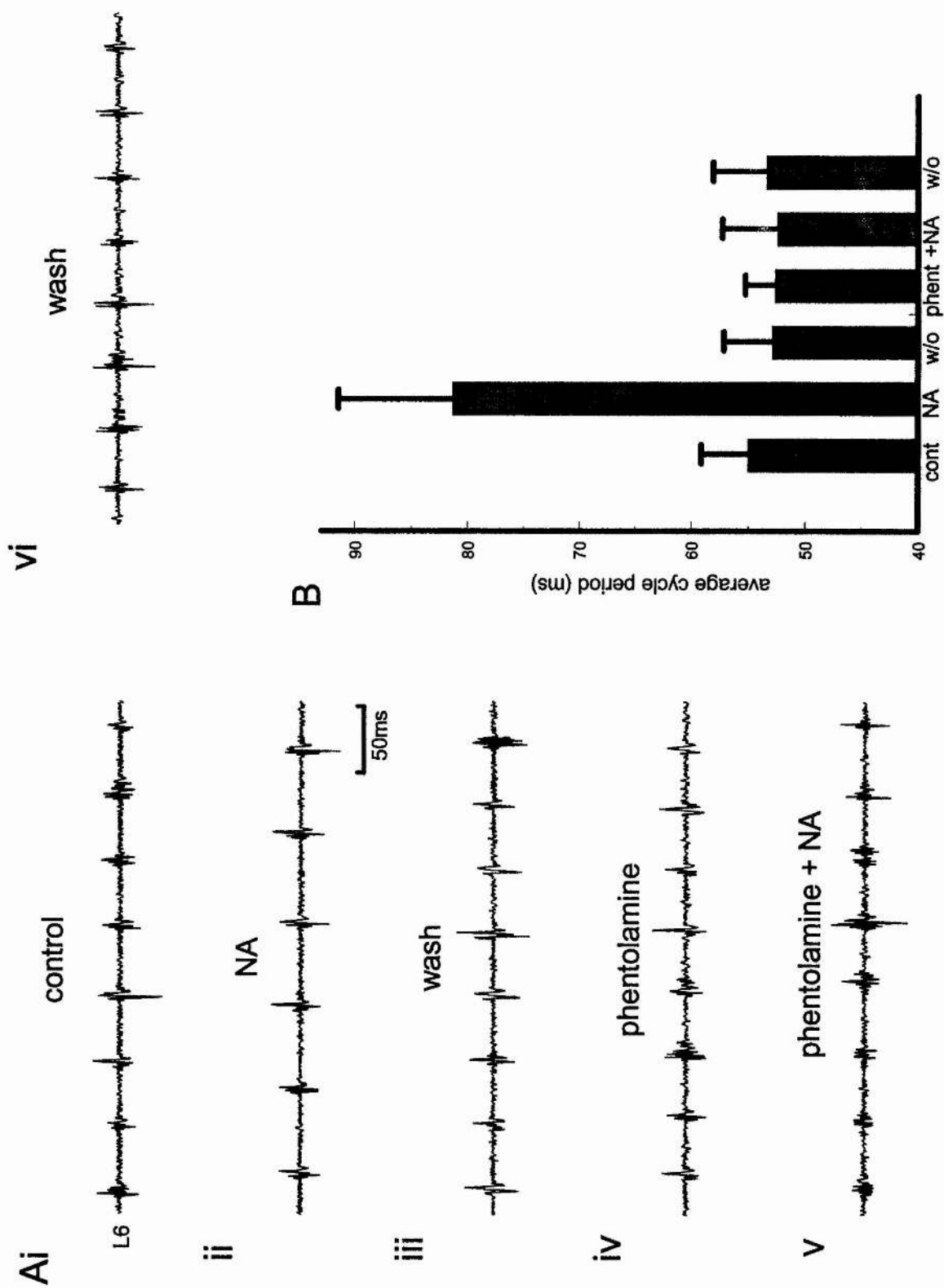


Figure 3.17. Phentolamine reverses the effects of NA on longitudinal co-ordination.

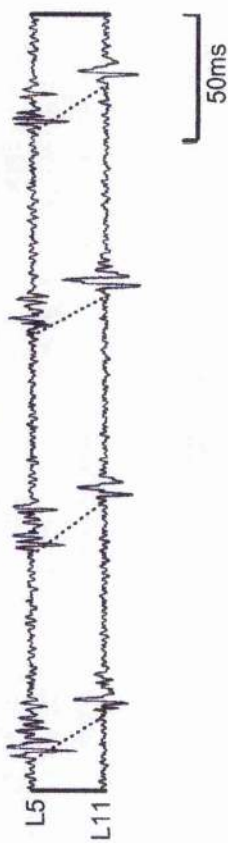
A. Exerpts of stage 42 larval fictive swimming activity recorded from L5 and more caudally from L11. Exerpts are frequency matched to show that longitudinal delays in control (i) are greater 10 minutes after addition of 5 μ M NA (ii). 20 minutes after subsequent addition of 50 μ M phentolamine, delays increase (iii). After 20 minutes wash in control saline, delays remain relatively long (iv).

B. Graph plotting delays against cycle period for complete episodes of swimming in control saline (n=232), 10 minutes after the bath application of 5 μ M NA (n=73) and 20 minutes after subsequent exposure to 50 μ M phentolamine (n=92). In control conditions, the correlation coefficient is highly significant ($p < 0.001$) with $r = 0.73$. After exposure to NA, the relationship between these two parameters of swimming shifts so that delays decrease whilst cycle periods increase. The correlation coefficient remains highly significant ($p < 0.001$) in the presence of the amine with $r = 0.71$. Subsequent exposure to phentolamine reverses the effects of NA, so that the delays increase, whilst the r-value remains at 0.71. Data taken from the same experiment shown in A.

C. Histograms representing the mean longitudinal delay under each experimental condition. In control conditions, mean delays are 10.0 ± 1.6 ms. Delays significantly ($p < 0.001$) decrease to 6.1 ± 2.3 ms 10 minutes after application of 5 μ M NA. Further addition of phentolamine increases delays to 9.1 ± 2.2 ms. Data taken from a different to those shown in A and B.

Ai

control



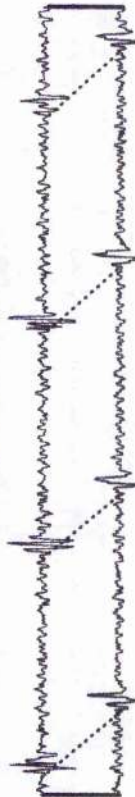
ii

NA



iii

NA + phentolamine

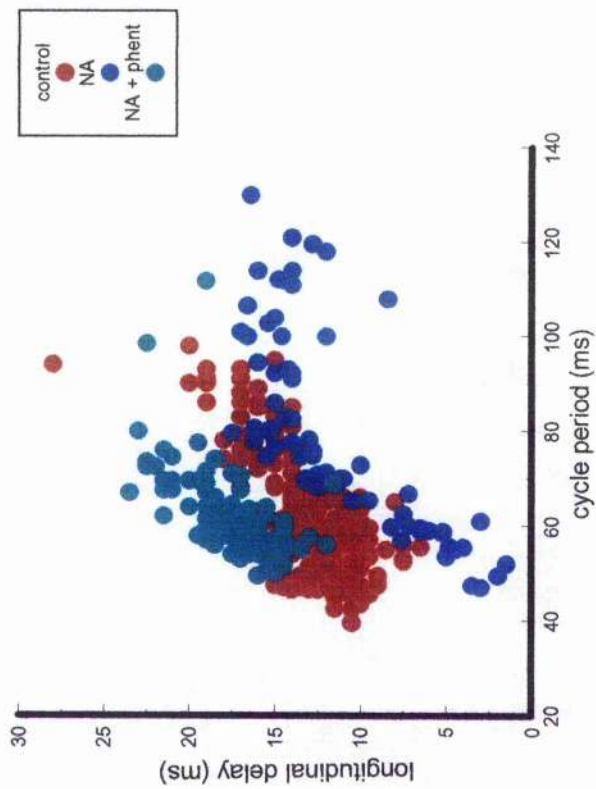


iv

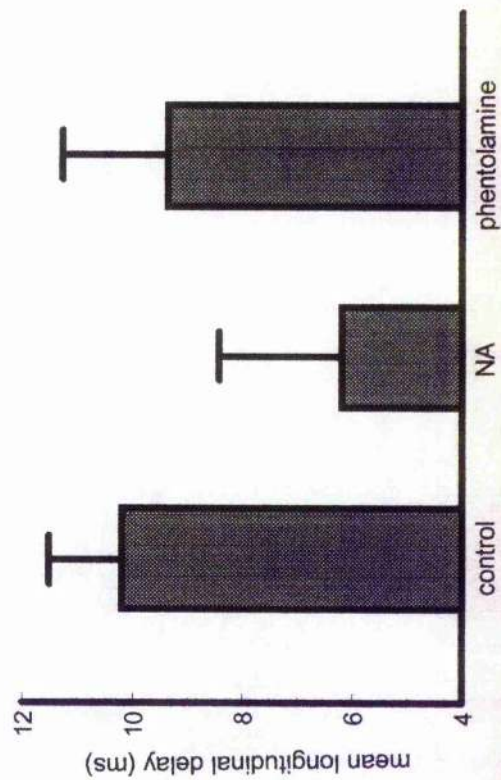
wash off phentolamine



B



C



exposure to the antagonist (figure 3.17C), suggesting that effects on longitudinal co-ordination may be mediated through α -adrenergic receptors.

One effect that was not reversed by phentolamine was the NA-mediated decrease in episode duration (figure 3.18). This finding indicates that NA-mediated effects on the duration of swimming episodes are not mediated through an action on α -like adrenoceptors.

When phentolamine was applied to stage 42 preparations that displayed a relatively slow, bursty motor pattern under control conditions (figure 3.19A), cycle periods during swimming episodes were reduced whilst delays were increased (figure 3.19B,C,D) twenty minutes after the bath application of the antagonist ($n=3$). This suggests that in these older, stage 42 preparations, endogenously released NA may actively control the frequency and longitudinal co-ordination of the swimming motor pattern. In all experiments, phentolamine was found only to be effective in preparations which had a region of myotomes removed to expose the underlying spinal cord, suggesting that care may have to be taken to ensure good spinal accessibility when using the antagonist.

The effects of phentolamine on the NA-mediated modulation of swimming frequency were also examined in stage 42 larvae that had been transected at the level of the first post-otic cleft, as shown in figure 3.20. NA did not significantly ($p<0.05$) increase cycle periods in transected animals in the presence of 50 μ M phentolamine. Furthermore, NA did not significantly affect delays in transected animals in the presence of phentolamine (figure 3.21). However, delays in transected larvae are not significantly affected when NA is added alone (see figure 3.13), so it may not be surprising that this is the case in the presence of the antagonist. Nevertheless, these findings indicate that either the effects of NA require the co-activation of both α_1 and α_2 receptors or that the amine is acting at a pharmacologically distinct type of receptor. Experiments

Figure 3.18. Phentolamine does not reverse the NA induced decrease in episode durations in stage 42 larvae

Histogram depicting episode durations averaged from three episodes of stage 42 larval fictive swimming under each experimental condition. Mean episode durations significantly ($p < 0.001$) decreased from 21.8 ± 5.3 sec in control to 4.5 ± 2.7 sec after the bath application of $5\mu\text{M}$ NA. Subsequent exposure for 20 minutes to $50\mu\text{M}$ phentolamine did not significantly ($p > 0.05$) increase episode durations, with the mean at 4.3 ± 1.4 sec.

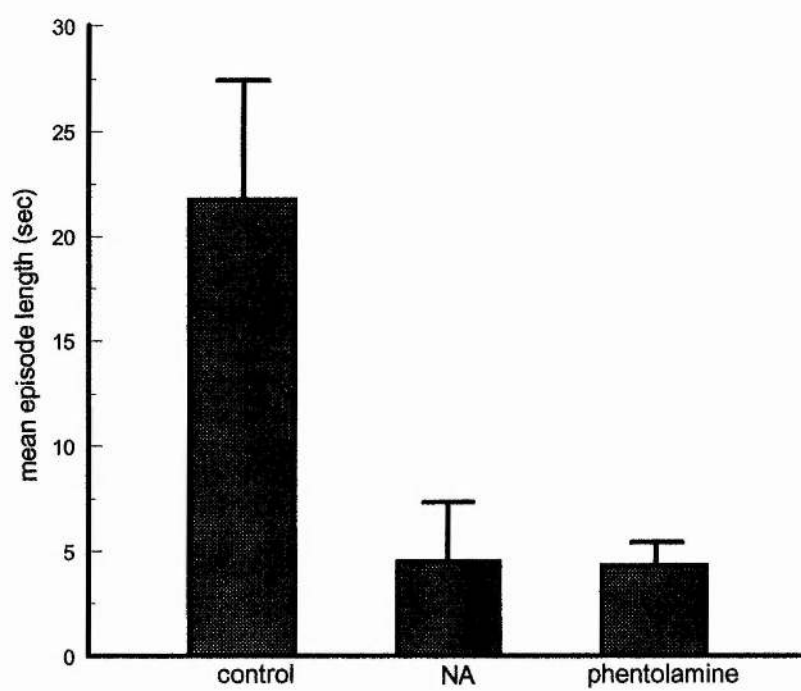


Figure 3.19. Phentolamine modulates fictive swimming in the stage 42 larva.

A. Exerpts of fictive swimming recorded from L4 and L12 in a stage 42 preparation and taken from near the start of an episode of fictive swimming in both control saline (i) and 20 minutes after the bath application of 50 μ M phentolamine (ii).

B. Histogram showing that phentolamine significantly ($p < 0.001$) decreases cycle periods from 55.7 ± 4.8 ms in control to 46.8 ± 4.9 ms after 20 minutes exposure to the antagonist.

C. Histogram demonstrating phentolamines ability to increase longitudinal delays during swimming. Delays increase from 12.5 ± 2.4 ms in control to 17.5 ± 2.5 ms after a 20 minute exposure to the antagonist (50 μ M).

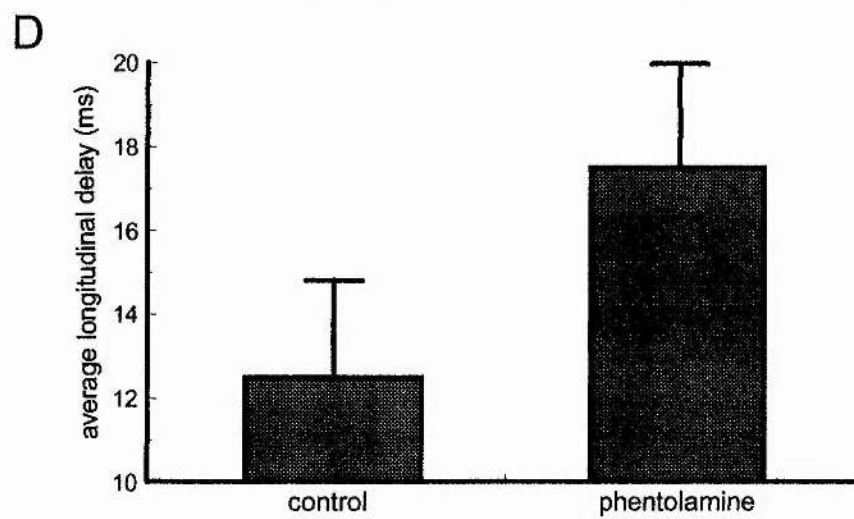
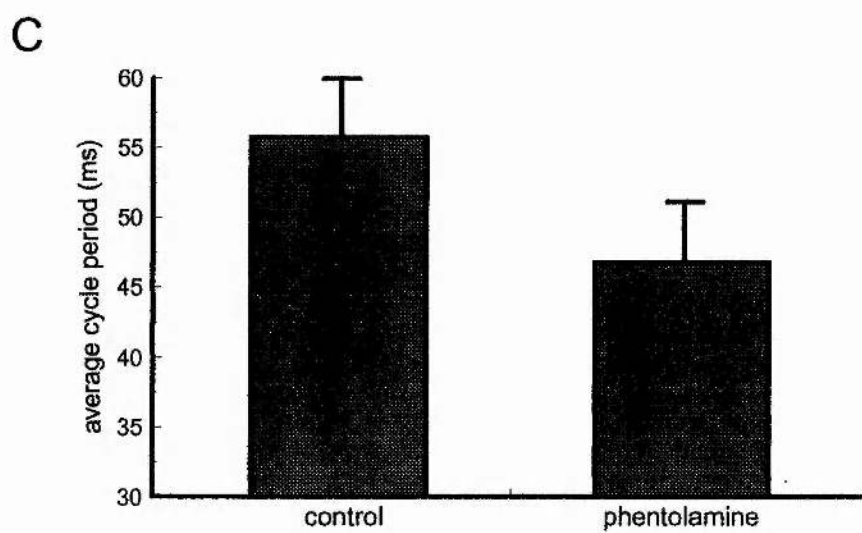
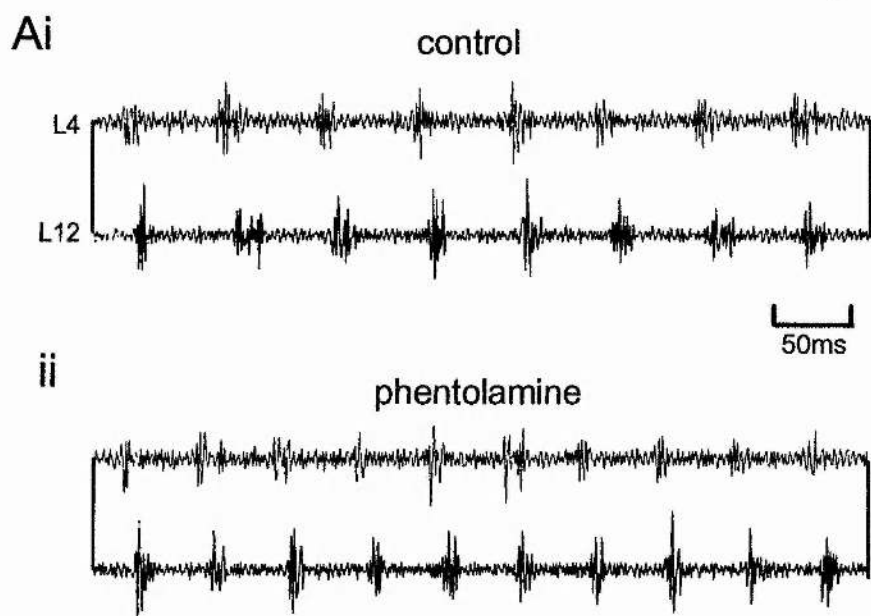


Figure 3.20. Phentolamine blocks the effects of NA on cycle period during swimming in larvae transected at the level of the first post otic cleft.

A. Stage 42 larvae were transected at the level of the first post otic cleft (approximately at arrow).

B. Exerpts of ventral root activity recorded from L5 and L12. Exerpts are recorded from equivalent points near the start of an episode of swimming in control (i), 10 minutes after the bath application of $1\mu\text{M}$ NA (ii), after around 25 minutes wash in control saline (iii), 20 minutes after the bath application of $50\mu\text{M}$ phentolamine (iv) and after subsequent addition of $1\mu\text{M}$ NA (v).

C. Histogram showing the mean cycle periods averaged from 30 consecutive cycles in three different episodes of swimming for each experimental condition. Cycle periods significantly ($p < 0.001$) increased from 67.5 ± 10.7 ms to 77.9 ± 10.4 ms after bath application of $1\mu\text{M}$ NA. Wash to control saline reduced average cycle periods to 55.7 ± 10.6 ms. After 20 minute exposure to $50\mu\text{M}$ phentolamine, cycle periods were on average 53.1 ± 8.8 ms. Subsequent re-addition of $1\mu\text{M}$ NA did not significantly ($p > 0.05$) affect cycle period durations, the mean being 55.5 ± 9.6 ms. Data in B and C are taken from the same experiment.

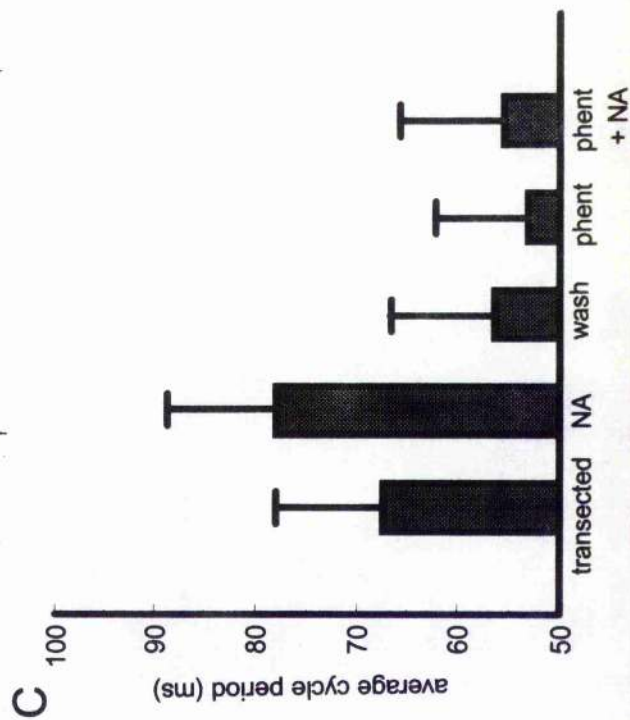
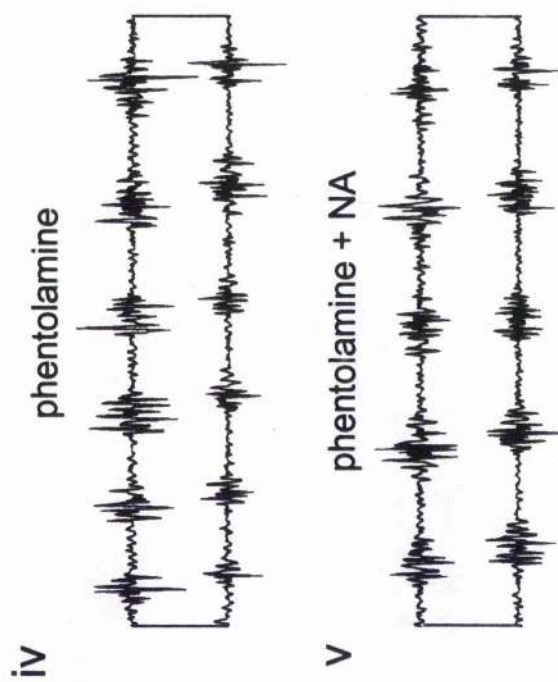
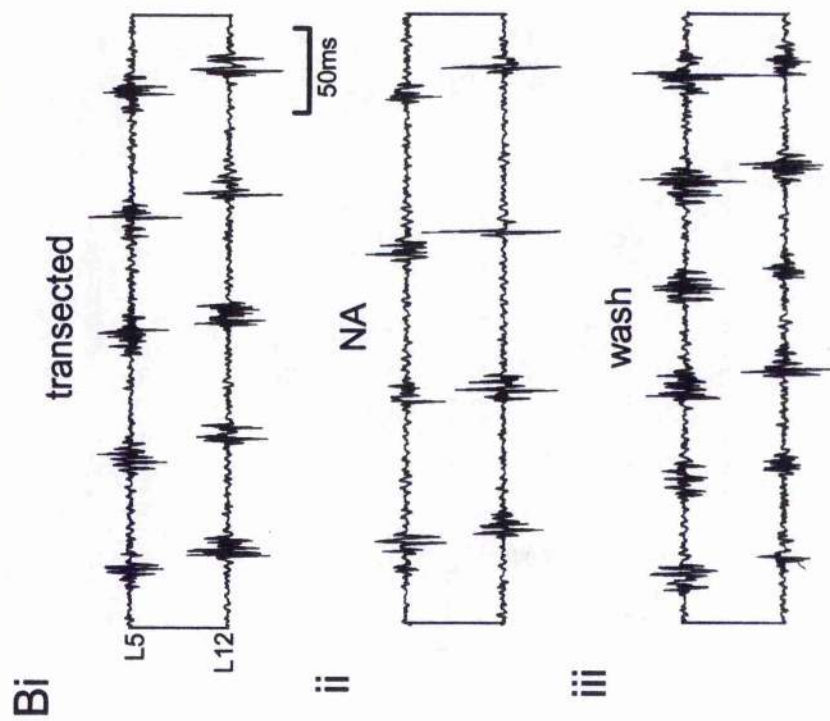
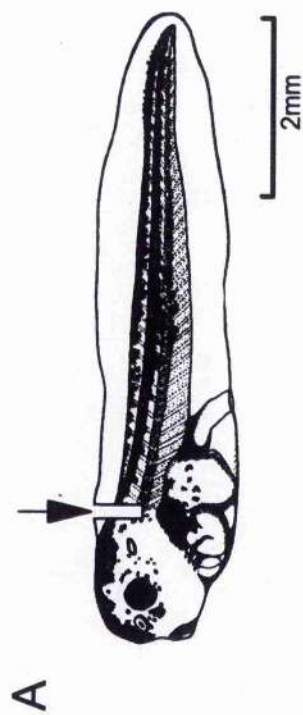
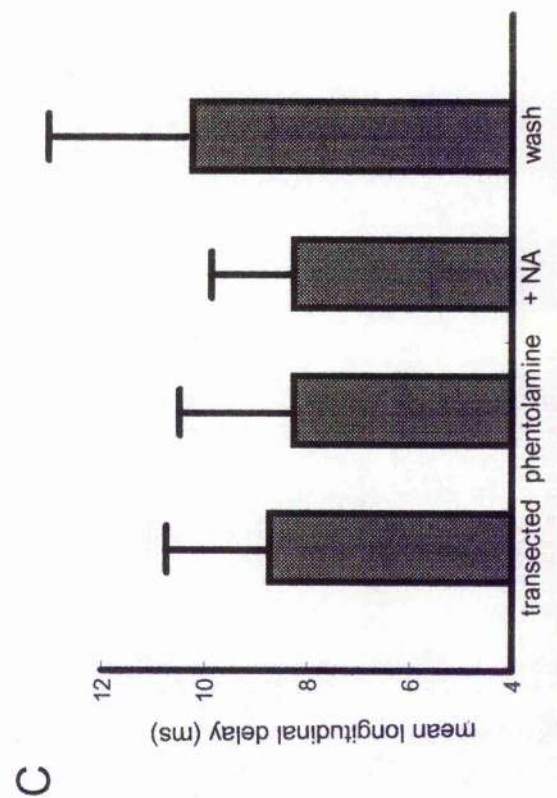
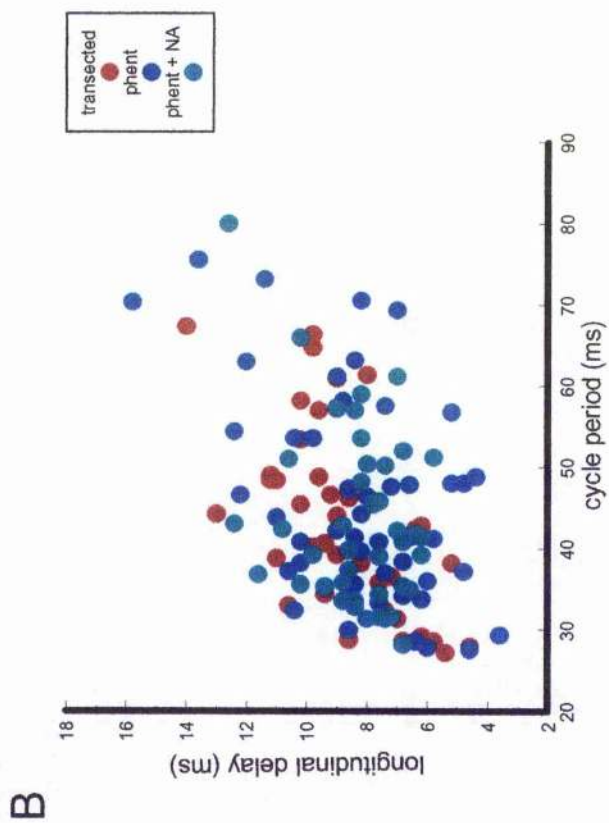
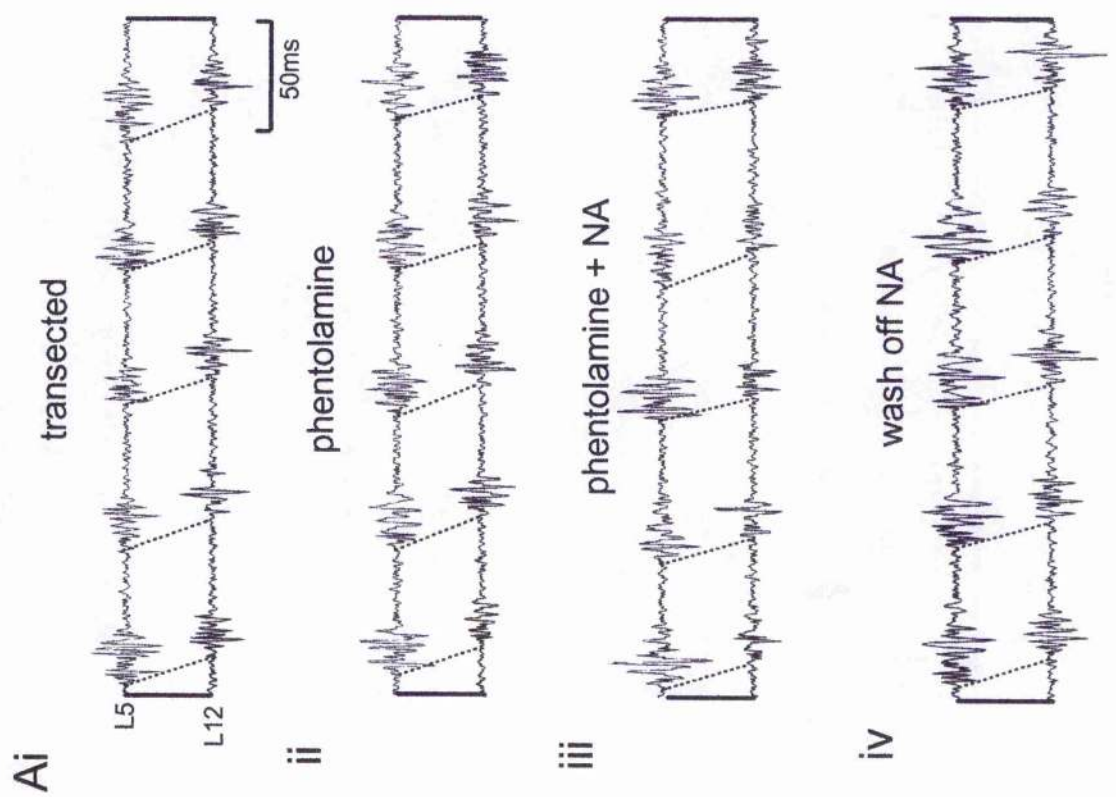


Figure 3.21. Effects of phentolamine and NA on longitudinal co-ordination in larvae transected at the level of the first post otic cleft.

A. Exerpts of transected stage 42 larval fictive swimming activity recorded from L5 and L12. Exerpts are frequency matched to show delays (depicted by dotted lines) in control (i), after addition of 50 μ M phentolamine (ii), after subsequent addition of 1 μ M NA (iii) and during wash (iv).

B. Graph plotting delays against cycle periods for three complete episodes of swimming in control transected animals (n=38), after addition of 50 μ M phentolamine (n=51) and after further addition of 1 μ M NA (n=44). In control conditions, the correlation coefficient is significant ($p < 0.001$), but the correlation is relatively weak with $r = 0.60$. After exposure to 50 μ M phentolamine, the relationship between these two parameters of swimming does not appear to be affected. The correlation coefficient is significant ($p < 0.001$) in the presence of phentolamine ($r = 0.47$). After 10 minutes exposure to 1 μ M NA in the presence of phentolamine, the correlation coefficient remains weak ($r = 0.28$), whilst there does not appear to be any effect on the relationship between delays and cycle period.

C. Histograms representing the mean longitudinal delay under each experimental condition. In control conditions, mean delays are 8.6 ± 2.1 ms. Delays do not significantly ($p > 0.05$) change in the presence of phentolamine (mean = 8.1 ± 2.4), or after further addition of 1 μ M NA (mean = 8.2 ± 1.7 ms). Delays increase to 10.9 ± 2.8 ms after 20 minutes in wash.



employing the use of a wider range of α receptor agonists and antagonists will help to determine the receptor type(s) involved in the NA response.

DISCUSSION

An inherent requirement of motor systems is that they are able to express some degree of flexibility in order to allow for adaptation to different behavioural contexts and developmental requirements. Changes in neuronal properties and synaptic connections are a potentially important source of this variability and furthermore, intrinsic modulation by endogenous neuroactive substances may play an important role in imparting such flexibility to the neural networks that form these locomotor systems. Such mechanisms may allow the animal to express a wide range of motor outputs, suited to its particular requirements. This chapter has demonstrated that the biogenic amine NA is one such modulator that is capable of profoundly affecting the output of the locomotor system of the *Xenopus* tadpole in several ways. These effects and their wider significance is discussed below.

i) Effects on swimming frequency.

Perhaps the most striking effect of NA on *Xenopus* tadpole locomotion is its ability to significantly lengthen cycle periods during fictive swimming (see figures 3.2 and 3.4). This reduction in rhythm frequency is similar to the effects seen in other vertebrates, suggesting that this function of NA may have been phylogenetically conserved during evolution. In the lamprey for example, NA has been shown to cause a slowing of the swimming motor pattern by around 50% (McPherson & Kemnitz, 1994). Furthermore, in addition to initiating walking in the chronic spinal cat (Fossberg & Grillner, 1973; Barbeau & Rossignol, 1991, Kiehn, Hultborn & Conway, 1992) noradrenergic drugs, such as the α_2 agonist clonidine, have been shown to reduce the frequency of motor output (Fossberg &

Grillner, 1973; Barbeau & Rossignol, 1991). Other central rhythm generating systems respond to NA in a similar way. The respiratory system of the new-born rat is one example, where it has been shown that NA causes a decrease in rhythm frequency (Errichidi, Hilaire & Monteau, 1990). This effect is potentiated by pargyline, a MAO inhibitor, and blocked by both the α_2 antagonist yohimbine and α -methyltyrosine, an inhibitor of NA biosynthesis. It would therefore appear that in other systems, the slowing of locomotor output is mediated through an action on α_2 receptor subtypes. Some NA-induced effects on motor output in *Xenopus* tadpoles are blocked by the broad spectrum α -receptor antagonist phentolamine, although further studies are required before the precise pharmacology of the response can be established.

ii) Effects on longitudinal co-ordination.

The effect of NA on longitudinal co-ordination during fictive swimming in *Xenopus* tadpoles was both profound and counterintuitive to the situation that is typical in these animals. In the stage 37/8 embryo, no relationship between longitudinal delay and cycle period exists, but, by stage 42, a correlation between rostrocaudal delay and swimming frequency has developed, so that as cycle periods lengthen, the magnitude of the longitudinal delays also increases (Tunstall & Sillar, 1993). This scaling of delays with cycle periods presumably allows the larvae to maintain an optimum body shape over a wide range of swimming frequencies. It would have therefore seemed reasonable to expect that, as NA causes a dramatic decrease in swimming frequency, a proportional increase in longitudinal delays would also be observed. However, bath application of NA has the opposite effect, in that at a given cycle period, the longitudinal delays were markedly shorter when compared to those in control conditions in both stage 37/8

and 42 animals. It is difficult to imagine how the resulting motor behaviour would fit into a useful behavioural context, as decreasing longitudinal delays would give rise to more rapid propagation of the flexions that occur down each side of the body within each cycle. Under these circumstances, if cycle periods are simultaneously increased, a delay in motor output would presumably occur during alternation between the two sides of the body, so that the animal effectively 'pauses' between output across each side. Perhaps the amine is important in generating the expression of other forms of behaviour: when sustained pressure or repetitive sensory stimulation is applied to *Xenopus* tadpoles, alternating waves of more prolonged ventral root bursts (150-300ms) occur which propagate along each side of the body in a caudo-rostral direction (Kahn & Roberts, 1982b; Soffe, 1991b, Green & Soffe, 1996) the reverse of that observed during normal fictive swimming. This activity, termed "fictive struggling" is characterised by repetitive firing of motoneurons on each cycle of activity (Soffe, 1991b; Kahn & Roberts, 1982b; Soffe & Roberts, 1982b; Soffe, 1993; Green & Soffe, 1996), which directly contrasts with the single impulses generated in motoneurons during swimming. This behaviour is thought to be activated through the same R-B sensory pathway as swimming since it can be generated in preparations that have been spinalised caudal to the cranial roots (Soffe, 1991b). Its expression also does not appear to depend on sensory discharge as rapid application of EAA agonists can evoke struggling (Soffe, 1996). Furthermore, recordings from premotor interneurons and motoneurons have shown that struggling appears to employ the same spinal circuitry as that utilised during normal swimming (Soffe, 1993; Green & Soffe, 1996), suggesting that the circuitry that generates motor output must be modified in some way in order to switch from swimming to struggling behaviour. How the neural circuitry is re-configured to allow for change from one distinct motor pattern to another is not as yet clearly understood, but perhaps neurotransmitters play a role. During struggling, cycle periods increase and delays

are reversed. Obviously bath application of NA does not induce struggling activity but it could reconfigure the spinal network, or some of its components, in a similar way to that which takes place during struggling. Perhaps when exposed to NA during swimming, the network is attempting to re-configure itself into struggling, causing occasional bouts of irregular ventral root discharge, long cycle periods and reduced (and sometimes reversed) rostrocaudal delays. NA has been shown to often be co-localised in large-core vesicles with many neuroactive substances such as opioid peptides (Wilson, Klein, Chang, Gasparis, Viveros & Yang, 1980), somatostatin (Hökfelt, Elfvin, Elde, Schultzberg, Goldstein & Luff, 1977), substance P (Kessler & Black, 1982) and neuropeptide Y (Lundberg, Terenius, Hökfelt & Goldstein, 1983). Perhaps release of NA, rather than causing the expression of struggling, helps to bias the motor pattern towards this form of behaviour. Peptides co-localised with NA may alter other properties of the motor network. Co-release of peptidergic transmitters in conjunction with NA may trigger the expression of struggling behaviour. It is interesting in this context that Rohn-Beard neurones, whose repetitive activation can trigger struggling show substance P-like immunoreactivity (Clarke, Hayes, Hunt & Roberts, 1984; Gallagher & Moody, 1987), although it is not known whether they co-localise NA. Struggling can be elicited in animals spinalised at the level of the first post otic cleft (Soffe, 1991b), which means that the expression of this behaviour does not require the presence of descending modulatory input from the brainstem. However, this does not rule out the possibility that chemical modulators, such as NA, are important for causing alterations of certain properties of the motor network in such a way that helps to cause the expression of struggling behaviour. With respect to this point, neuromodulation has been suggested to be a means through which a single neural network can be induced to express several different forms of behaviour, a topic which will be addressed in chapter 5.

iii) Effects on threshold for skin stimulation.

Another reliable effect of NA at both stages examined was an increase in threshold for skin stimulation. NA has been shown to modulate sensory transmission in other vertebrates, via effects in the dorsal horn (see introduction) and it may be that the amine exerts analogous effects in the spinal cord of *Xenopus* tadpoles. There are known to be two sensory pathways in the embryo, the mechanosensory Rohn-Beard pathway and the skin cell sensory pathway (Clarke, Hayes, Hunt & Roberts, 1984; Roberts & Smyth, 1974). It is possible that the observed increase in threshold for skin stimulation seen after NA exposure is mediated through an inhibitory action on one or both of these pathways. The skin cell pathway is thought to access the CNS through the trigeminal nucleus (Roberts, 1996), so transecting at the level of the first post otic cleft should effectively remove skin sensory input. My experiments performed on stage 42 larvae transected at this level, showed that NA was still capable of having a potent effect on the threshold for skin stimulation. This suggests that the Rohn-Beard cell pathway at least is inhibited by application of NA. Further experiments are clearly needed to elucidate the role NA plays in any possible modulation of this pathway.

iv) The ontogeny and pharmacology of noradrenergic systems.

The profound influence of NA on *Xenopus* tadpole spinal sensory and motor circuitry suggests the presence of a functional noradrenergic system at an early stage in the animals life. The primary effect of NA on swimming is to cause a slowing of the motor rhythm. This effect of NA is apparent at both stages 37/8 and 42, suggesting that receptors for NA are present within *Xenopus* tadpoles at

least as early as late embryogenesis. The earliest stage examined for possible NA localisation in *Xenopus* is stage 38 where it was shown that spinal tyrosine-hydroxylase immunoreactive neurones are present in the CNS by this stage in development (Gonzalez, Marin, Tunihoff & Smeets, 1994). Some of these neurones have fibres projecting to the spinal cord, and it may be that a proportion of them synthesise NA. Furthermore, a recent study using high performance liquid chromatography has shown that NA is detectable in the body of the tadpole at around the time of hatching and gradually increases in concentration during larval development (Kloas, Reinecke & Hanke, 1997). The electrophysiological evidence presented here points towards the presence of NA receptors at least as early as stage 37/8. The NA receptors that mediate effects on motor output are likely to be located within the spinal cord, since NA, at least in stage 42 larvae, still has a marked effect on swimming in animals transected at the level of the first post-otic cleft. The fact that phentolamine was capable of blocking the effects of NA points towards a role for an α -like receptor in mediating the effects of NA on the motor pattern. However, the effects of other α antagonists (including selective α_1 and α_2 antagonists), and β antagonists needs to be studied in more detail before the receptor type involved can be identified. One of the current problems of studying noradrenergic systems is that relatively few selective pharmacological agents for adrenergic receptors are available. Furthermore, of the ones that do exist, although their activity is generally well characterised in mammals, very little is known of their affinity and efficacy at non-mammalian noradrenergic receptors. The lack of effect of the bulk of adrenergic agents tested here (such as phenylephrine and clonidine) may highlight differences between the receptor pharmacology of amphibians and mammals.

The indolamine 5-hydroxytryptamine (5-HT) has previously been shown to induce effects which contrast with those reported here for NA: 5-HT causes a strong, bursty pattern of motor discharge which results in a large proportion of

each cycle being occupied by ventral root activity (Sillar Wedderburn & Simmers, 1992) while longitudinal delays are increased under 5-HT (Tunstall & Sillar, 1993). NA has the opposite effect so that a very small proportion of each cycle of activity is occupied by ventral root discharge, whilst longitudinal delays are decreased. It may be that by controlling the degree to which endogenous NA and 5-HT are released within the spinal cord during swimming the frequency, intensity, and longitudinal co-ordination of swimming activity can be precisely adjusted. A descending influence on the spinal circuitry for locomotion imposed by such modulatory systems may provide the animal with the flexibility it needs to allow adaptation to behavioural and environmental requirements.

v) Final comments.

In this chapter I have shown that NA has a marked ability to affect the motor output of both *Xenopus* embryos and larvae. The primary effects of the amine are a slowing of the swimming frequency and a reduction in rostrocaudal delays. Although pharmacological characterisation is incomplete, α -adrenergic receptors appear to play a role in mediating the effects of NA, as the broad-spectrum α -antagonist phentolamine can reverse the effects of the amine on motor output. The effects on swimming frequency can also be reproduced in larvae that have been transected at the level of the first post otic cleft, suggesting that NA is acting on receptors within the spinal cord to exert this effect. These findings strengthen the premise that the amine NA is an important modulator of spinal locomotor networks.

CHAPTER 4

Noradrenaline strengthens inhibitory synapses in the *Xenopus* tadpole spinal cord

INTRODUCTION

i) Background.

It has long been known that synaptic inhibition is an important component of the neural networks that underlie rhythmic behaviours. As far back as 1911 it was suggested that inhibition plays a role in co-ordinating antagonistic "half-centres" in the mammalian spinal cord by ensuring alternation between flexor and extensor activity during locomotion (Brown, 1911). Such inhibition is thought to co-ordinate activity within most neural networks for rhythmic motor behaviour (such as mastication, walking and swimming) ensuring that antagonistic motor pools are always active in strict antiphase.

There are two candidate transmitters that have been implicated in mediating reciprocal inhibition within vertebrate rhythm generating systems. These are the amino acids GABA and glycine (see Kiehn, Hounsgaard & Sillar, 1997 for review). GABA is the main inhibitory neurotransmitter within the vertebrate brain, exerting its effects through GABA_A, GABA_B and GABA_C receptor subtypes. Of these, GABA_A and GABA_C receptors are ionotropic whilst GABA_B receptors are metabotropic. The involvement of GABA in motor control has been examined in some detail. In the cat, for example, the transmitter presynaptically inhibits sensory afferents in the spinal cord, ensuring that sensory information does not interrupt rhythmic motor behaviour during locomotion (Dubuc, Cabelguen & Rossignol, 1988). GABA may also play other important modulatory roles during locomotion: in the lamprey, spinal interneurons are thought to release GABA onto motoneurons, causing membrane potential oscillations during locomotion. This effect is generated by both GABA_A and GABA_B receptors (Alford, Christenson & Grillner, 1990). GABA_A receptors also

appear to presynaptically assist in motoneurone repolarisation following phasic excitation of lamprey motoneurons, especially in the absence of glycinergic inhibition (Alford, Sigvardt & Williams, 1990). With respect to behavioural effects of the transmitter, the amino acid appears to be important for regulating swimming frequency, affecting longitudinal co-ordination and stopping locomotion in the spinal cord of this animal (Tégner, Matsushima, El Manira & Grillner, 1993). An increase in GABAergic inhibition also regulates both motor frequency and termination of motor output in the neonatal rat (Cazalets, Sqalli-Houssaini & Clarac, 1994). In *Xenopus* tadpoles, 5 β -pregnan-3 α -ol-20-one, a positive allosteric modulator of many vertebrate GABA_A receptors (see Reith, 1996 for review) causes a decrease in swimming frequency and the duration of swimming episodes (Reith, 1996). Ventral root burst amplitude and duration of *Xenopus* embryo swimming episodes have been shown to be reduced by baclofen, an agonist of GABA_B receptors. Intracellular recordings have demonstrated that baclofen both reduces reciprocal glycinergic inhibition and increases the spike threshold of motoneurons during swimming (Wall & Dale, 1993). GABA also appears to play an important, but temporary role in spinal motor control during early stages of development in the chick embryo. In the spinal cord of these animals, inhibitory GABAergic input occurs onto flexor motoneurons thereby restricting their activity early in development. This GABAergic input is lost at a later stage, allowing more precise control over the timing of motoneurone firing to be attained (O'Donovan, Sernagor, Sholomenko, Ho, Antal & Yee, 1992). There is therefore a growing body of evidence to suggest that GABA is a modulator of neural networks for motor control, but is it essential for rhythm generation? In the lamprey blockade of GABA_A receptors with bicuculline does not abolish reciprocal inhibition (Grillner & Wallén, 1980). A similar effect has been reported in the *Xenopus* embryo (Soffe, 1987). Initial reports in the neonatal rat preparation also supported these observations (Cazalets, Sqalli-Houssaini &

Clarac; 1994). However, a more recent finding has shown that bicuculline completely blocks reciprocal inhibition in the neonatal rat (Cowley & Schmidt, 1995).

Whilst the role of GABAergic inhibition in spinal rhythm-generation requires further investigation, much more is known of the function of glycinergic inhibition. Glycine, a small amino acid found in abundance throughout the grey matter of the spinal cord and the brainstem, acts as the ligand for a chloride conducting ion channel that is functionally similar to, but pharmacologically distinct from the GABA_A receptor. Glycinergic inhibition is largely responsible for mediating the reciprocal organisation of antagonistic motor pools within the spinal cord (Jordan, 1983). In the lamprey and the *Xenopus* embryo, the source of glycinergic inhibitory input to motoneurons has been studied in much detail. In both animals reciprocal inhibition arises from populations of glycinergic interneurons which have axons that project to the opposite side of the spinal cord (Dale, 1985, Buchanan, 1982; Buchanan & Cohen, 1982; Buchanan & Grillner, 1987). In the *Xenopus* embryo, these neurons are called the commissural interneurons, and are the sole class of glycine-immunoreactive cells within the entire CNS (Dale, 1985; see below). In the lamprey, similar neurons are called the 'CC' interneurons, although other types of glycinergic cell have been shown to exist: inhibitory interneurons with ipsilaterally projecting axons that are immunoreactive for glycine have also been identified which may be active during locomotion, contributing to the final output of the motor network (Buchanan & Grillner, 1988). Very little is currently known of the source of spinal glycinergic inhibition in higher vertebrates. Nonetheless, both neonatal rat (Kjærulff & Kiehn, 1997) and cat (Perret, 1983; Noga, Cowley, Huang, Jordan & Schmidt, 1993) motoneurons receive strychnine sensitive, Cl⁻ dependent ipsps during locomotion. In the rat, this inhibition appears to arise from interneurons whose axons cross to the opposite side of the spinal cord since activation of cells in one

half of the cord (with 5-HT and NMDA) induces rhythmic ipsps in motoneurones in the opposite side of the cord (Kjærulff & Kiehn, 1997).

ii) Reciprocal inhibition in the stage 37/8 *Xenopus* embryo.

In stage 37/8 *Xenopus* embryos, the neural networks that co-ordinate locomotion are organised as antagonistic half-centres that are coupled by reciprocal inhibition that occurs 'mid-cycle' during swimming activity. The anatomy, physiology and pharmacology of the interneurones that generate such inhibition has been studied extensively, contributing much to our understanding of the function of reciprocal inhibition within networks for rhythm generation. Immunocytochemical studies have revealed that in the entire CNS of the *Xenopus* embryo, a single class of neurone stains with antibodies against glycine (Dale, Otterson, Roberts & Storm-Mathisen, 1986). Neurones of this type, termed 'commissural interneurones', begin to appear at a very early stage in development (at around stage 20-22 (Jacobson & Huang, 1985; Roberts, Dale, Ottersen & Storm-Mathisen, 1988)). By stage 37/8, they are found distributed along the length of the spinal cord extending rostrally into the brainstem (Dale, Otterson, Roberts & Storm-Mathisen, 1986). There are estimated to be around 600 of these cells in the stage 37/8 embryo CNS. Their somata lie just dorsal to the mid-level of the spinal cord. Their axons project beneath the neurocoel to the opposite side of the spinal cord where they T-branch and ascend towards the midbrain and descend to within 600µm of the caudal tip of the spinal cord. A small proportion of the commissural interneurones also possess ipsilaterally projecting axons (Roberts, Dale, Ottersen, & Storm-Mathisen, 1988, see below).

Neuroanatomical studies have therefore shown that the commissural interneurones are present within the spinal cord of the embryo. This raises the

question as to whether the spinal circuitry of the CPG is sensitive to glycine. Electrophysiological studies have revealed that spinal motoneurons hyperpolarise in response to bath application of glycine, an effect which is blocked by strychnine (Soffe, 1987). The reversal potential of this response is close to the resting membrane potential of the motoneurons but can be made strongly depolarising by using KCl as the electrolyte (Soffe, 1987). Presumably this is because KCl leaks into the cell, thereby artificially elevating intracellular Cl^- levels and changing the Cl^- equilibrium potential to a more depolarised level. Although the effects of glycine on other elements of the CPG are not known, the studies outlined above are consistent with the premise that postsynaptic chloride-conducting glycine receptors are located on spinal motoneurons.

Is there an endogenous source of glycine released onto motoneurons within the spinal cord? Paired intracellular recordings from commissural interneurons and motoneurons have shown that impulses evoked in commissural interneurons following depolarising current injection evoke short-latency ipsp's in contralateral (and in a small proportion of cases, ipsilateral) motoneurons (Dale, 1985). These potentials are strychnine-sensitive, and as such, are likely to be glycinergic. Therefore, inhibitory glycinergic synapses seem to be formed between the commissural interneurons and spinal motoneurons. The large majority of these connections are with contralateral motoneurons, although a few ipsilateral synapses are also formed.

There is strong experimental evidence to suggest that glycinergic inhibition forms an important component of the synaptic drive during swimming. All rhythmically active neurons -including motoneurons (Roberts & Kahn, 1982, Soffe & Roberts, 1982b), commissural interneurons (Soffe, Clarke & Roberts, 1984; Dale, 1985) and excitatory interneurons (Dale & Roberts, 1985)- receive mid-cycle inhibition during swimming. This component of the synaptic drive comprises a compound ipsp that is abolished by strychnine and becomes

strongly depolarising when recorded with KCl-filled electrodes (Roberts & Kahn, 1982; Soffe, 1987). Since the commissural interneurons are the only glycine-immunoreactive cells in the embryo CNS and since they are known to form inhibitory strychnine-sensitive connections with motoneurons on the opposite side of the cord, it is likely that these cells are the sole source of reciprocal mid-cycle inhibition during swimming. The hypothesis that mid-cycle inhibition arises from cells with contralateral projections comes from demonstrations that removing the excitatory drive to one side of the spinal cord (via rostral hemisection of that side) weakens mid-cycle inhibition in the other side (Soffe and Roberts, 1982a). The role of mid-cycle inhibition is probably to ensure the antagonistic coupling between the two sides of the spinal cord during swimming. During the excitatory phase of the swimming cycle in one side of the cord, commissural interneurons will fire and inhibit cells on the opposite side and vice versa, thereby ensuring strict alternation of motoneuron firing during swimming.

As stated above, a small proportion of commissural interneurons generate glycinergic ipsps in motoneurons on the same side of the spinal cord (Dale, 1985). These ipsps presumably arise from the sub-population of commissural interneurons that have ipsilaterally projecting axons (Roberts, Dale, Ottersen, & Storm-Mathisen, 1988). Small 'on-cycle' ipsps have also been reported to occur during swimming in a proportion of motoneurons (Clarke & Roberts, 1984; Sillar & Roberts, 1992b; Sillar & Roberts, 1993; Perrins & Soffe, 1996a) and dorsolateral commissural sensory interneurons (Sillar & Roberts, 1992; Soffe, 1993). Further evidence for the existence of ipsilateral inhibition comes from the finding that when recordings are made from motoneurons using KCl-filled electrodes, and amino acid and cholinergic inputs to motoneurons are weakened locally by microperfusion of kynurenate and dihydro- β -erythroidin respectively, a small on-cycle component remains. In some cells, part of this component is

strychnine sensitive (Perrins & Soffe, 1996a), and is therefore likely to be sign-reversed glycinergic inhibition. The remainder of the component is presumably electrotonic as it is not blocked by Cd^{2+} (see chapter 1). The role of on-cycle inhibition during swimming is not clear. It may limit multiple firing and regulate epsp amplitude, although there is currently no direct evidence for this. One report has, however, implicated ipsilateral inhibition as being essential for rhythm generation. Preparations with sagittally divided spinal cords (to remove mid-cycle inhibition) can still generate rhythm in one half of the cord. Subsequent exposure to strychnine greatly impairs rhythm generating capability, suggesting that it may rely on ipsilateral inhibition (Soffe, 1989). However, in direct contrast to this finding, the application of strychnine to intact preparations -to block both mid and on-cycle inhibition- does not abolish rhythm-generating capability. The role of ipsilateral inhibition during swimming awaits further clarification.

A notable feature of reciprocal inhibition in the embryo spinal cord is that it decreases in strength rostrocaudally. In areas rostral to the eighth post otic intermyotomal cleft, mid-cycle inhibition commonly occurs on all cycles of swimming activity and is of relatively uniform amplitude. Caudal to this level, ipsp tend to be smaller and even fail on some cycles. According to the findings of Tunstall and Roberts (1994), caudal to the twelfth post-otic cleft, motoneurones do not receive any glycinergic inhibition at all during swimming. This gradient in ipsp amplitudes could be causal in generating the rostrocaudal delays that occur in the timing of motoneurone firing during embryo swimming (Tunstall & Roberts, 1991, see discussion).

iii) Reciprocal inhibition in the stage 42 *Xenopus* larva.

Compared to the embryo, less is known about the organisation and function of commissural interneurons in stage 42 larvae. It has been shown that glycine-immunoreactive cells of a similar morphology to the commissural interneurons of the embryo are present in the spinal cord of stage 42 larvae (A.M. Woolston, unpublished observations). Electrophysiological experiments have also shown that motoneurons in the spinal cord of larvae receive prominent mid-cycle ipsp's during fictive swimming. This inhibition is both strychnine sensitive and reversed to become strongly depolarising when KCl is used as the electrolyte (Sillar, Simmers & Wedderburn, 1992). However, the inhibitory drive mid-cycle is generally much more complex compared to the embryo in that it comprises trains of ipsp's rather than a single compound event (see chapter 1). It is currently unknown as to whether on-cycle inhibition occurs in larval motoneurons, or whether there is a longitudinal gradient in the amplitude of inhibition within the spinal cord. Nonetheless, mid-cycle inhibition during swimming in the larvae has obvious parallels with that of the embryo and is likely to originate from the same population of glycinergic commissural interneurons that generate this component of the synaptic drive in the embryo.

iv) Is reciprocal inhibition essential for generation of rhythmic motor output?

Studies investigating the role that reciprocal inhibition plays in rhythm generation have employed two basic techniques: surgical removal of spinal reciprocal connections and pharmacological block of mid-cycle inhibition with the glycine receptor antagonist strychnine. The results produced under these conditions have been somewhat inconsistent. Rhythm generation has been shown

to occur within one half of the sagittally divided spinal cord of the embryo, where there can be no reciprocal inhibitory input present (Kahn & Roberts, 1982a; Soffe, 1989). Alternation between the two sides of the spinal cord is, however, abolished in such preparations (Kahn & Roberts, 1982a; Soffe, 1989). In contrast to these findings, in the lamprey, both the selective photo-ablation of CC interneurons and surgical hemisection of the spinal cord completely abolishes rhythm generating capability (Buchanan, 1996a, 1996b). The effects of strychnine in the lamprey depend on the concentration used. Low concentrations abolish neither rhythm generating capability nor alternation of motor output between the two sides of the spinal cord (Grillner & Wallén, 1980; Cohen & Harris-Warrick, 1984; McPherson, Buchanan & Kasicki, 1994). Intermediate doses, however, completely disrupt left-right co-ordination but do not abolish rhythm generating capability (Alford & Williams, 1987, 1989; Alford, Sigvardt & Williams, 1990; Cohen & Harris-Warrick, 1984). This gives rise to a synchronous motor pattern termed 'fictive galloping' which is thought to occur through a weak excitatory coupling between the two sides of the cord (Hagevick & McClellan, 1994). Higher doses of the antagonist completely disrupt rhythm generation in the lamprey (Grillner & Wallén, 1980). In limbed vertebrates, such as the neonatal rat (Cowley & Schmidt, 1995), adult cat (Noga, Cowley, Huang, Jordan & Schmidt, 1993) and wallaby (Ho, 1997) rhythmic alternation between two limbs and flexor-extensor muscle activity within a single limb can be disrupted by relatively high concentrations of strychnine, resulting in the production of bilaterally synchronous bursts of ventral root output.

Taking all these reports into consideration, it appears that glycinergic inhibition may well be important for ensuring alternation between the two half-centres of vertebrate CPGs. It is presently not clear as to whether it is essential for rhythm generation. One point worthy of note here is that strychnine is a rather non-specific drug that, aside from blocking glycine receptors, also blocks several

types of ion channel including those that generate K^+ currents and Ca^{2+} currents (Oyamo, Akaike & Carpenter, 1988; see also Dale, 1995b). It may be that high concentrations of strychnine used in the experiments discussed above affect rhythm generation through non-specific mechanisms rather than a direct action on glycine receptors.

v) Possible roles for inhibition in the control of rhythmic motor output.

Although there is still controversy over whether glycinergic inhibition is essential for rhythm generation, experimental evidence has indicated that alterations in the strength of glycinergic inhibition may help to shape the output of the neural networks for locomotion. For example, pharmacological block of glycinergic inhibition with strychnine in *Xenopus* tadpoles (Dale, 1985; Dale, 1995b; McDearmid, Scrymgeour-Wedderburn & Sillar, 1997) and in the lamprey (Cohen & Harris-Warrick, 1984; Grillner & Wallen, 1980; McPherson, Buchanan & Kasicki, 1994) accelerates swimming frequency. There is evidence to suggest that such effects may not be achieved solely through block of reciprocal inhibition as strychnine is still capable of accelerating motor output in one half of the *Xenopus* embryo spinal cord (Soffe, 1989). Since all cross-cord connections will have been removed in these preparations, the change in swimming frequency in this case must occur through mechanisms independent of reciprocal inhibition. Perhaps ipsilateral glycinergic inhibition is important for setting swimming frequency under these conditions. Nonetheless, the most comprehensive computer simulation study of *Xenopus* embryo swimming to date has supported the premise that the strength of glycinergic inhibition can profoundly affect locomotor frequency (Dale, 1995b). In this simulation, which incorporates experimentally-derived values for the biophysical properties of spinal neurones within the motor

network, the frequency of simulated swimming is dependent on the size/magnitude of the inhibitory conductance occurring mid-cycle. The model predicts that larger inhibitory conductances produce slower swimming frequencies whereas smaller inhibitory conductances produce faster swimming frequencies.

In embryos of the amphibians *Rana*, *Bufo* and *Tritus*, strychnine locally applied to spinal motoneurons gives rise to an increased reliability of cell firing during swimming. In *Rana* and *Bufo*, this effect is accompanied by an increase in burst durations recorded in nearby ventral roots (Perrins & Soffe, 1996a). During the course of this study, the investigators also reported that strychnine increased interneurone firing reliability in *Xenopus* embryos. No effect was seen on the firing properties of motoneurons in these animals, presumably because they tend to fire consistently on every cycle of swimming under control conditions. Strychnine did, however, advance the timing of motoneurone spiking on each cycle of activity in *Xenopus* and, to a lesser extent, *Rana* embryos (Perrins & Soffe, 1996a). The effect of local strychnine application on longitudinal co-ordination directly contrasts with the findings of Tunstall and Roberts in 1991 which indicated that during NMDA induced swimming, strychnine applied locally to caudal motoneurons increases rostrocaudal delays. The reasons for these discrepancies are not clear (see discussion).

Although findings differ between laboratories, the observations outlined above do have some interesting implications for the role of glycinergic reciprocal inhibition in shaping the final output of motor networks. They suggest that through alterations in the strength of this parameter of the synaptic drive, animals may be able to regulate the frequency, strength and co-ordination of motor activity to suit any contingency. If alterations in the strength of reciprocal inhibition are indeed an important means of imparting flexibility to the motor pattern, then this component of the synaptic drive may be susceptible to the

influence of neuromodulators such as NA. This idea provided the impetus to look at the effects of NA on glycinergic inhibition during fictive swimming in motoneurons of *Xenopus* tadpoles. In the course of these studies I obtained evidence that i) NA can increase the amplitude of ipsps during fictive swimming. ii) this effect of NA is, at least in part, mediated through an enhanced probability of glycine release from commissural interneurons rather than through a postsynaptic mechanism. iii) the slowing effect of NA on the motor pattern and its effects on longitudinal co-ordination can be largely explained by the amines enhancement of mid-cycle inhibition.

RESULTS

i) NA enhances mid-cycle inhibition in the stage 37/8 embryo.

In order to study the effects of NA on mid-cycle inhibition in *Xenopus* tadpoles, ventrally located neurones that were rhythmically active during fictive swimming (presumed to be motoneurones) were recorded with KCl filled microelectrodes. As a result, the glycinergic mid-cycle ipsp's were reversed in sign and enhanced in amplitude to become strongly depolarising (asterisked in figure 4.1Ai, ii; Roberts & Kahn, 1982; Soffe, 1987), making examination of evoked ipsp amplitudes easier. For the purposes of this study, cells positioned between the levels of the 3rd and 6th post-otic clefts are referred to as being 'rostral' whereas those located between the 12th and 16th post otic clefts are referred to as being 'caudal'.

Recordings made from presumed motoneurones in rostral regions of the embryo spinal cord supported previous findings that, unlike many other vertebrate motoneurones, they receive only a single compound epsp and ipsp during each cycle of swimming activity. Furthermore in this region of the spinal cord, the synaptic drive to motoneurones is typically strong on all cycles of activity within an episode of swimming so that reciprocal inhibition (asterisked in figure 4.1Ai) tends not to fail. When NA (1-10 μ M) was bath applied to such motoneurones (n=7), the amine was found to reversibly enhance the amplitude and reduce the variability of mid-cycle inhibition. The example shown in figure 4.1 is from a rostral motoneurone located at the level of the 4th post otic cleft. 8 minutes after the bath application of 2 μ M NA, mid-cycle ipsp's (asterisked in figure 4.1Ai,ii) became less variable and of generally higher amplitude. The overlaps of 16 consecutive ipsp's taken from towards the end of the episode in figure 4.1B show

Figure 4.1. NA enhances rostral mid-cycle inhibition in the embryo.

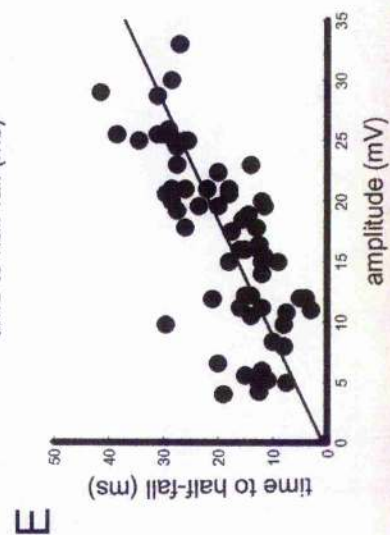
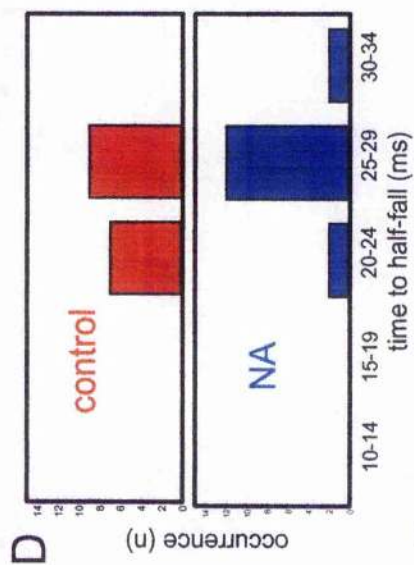
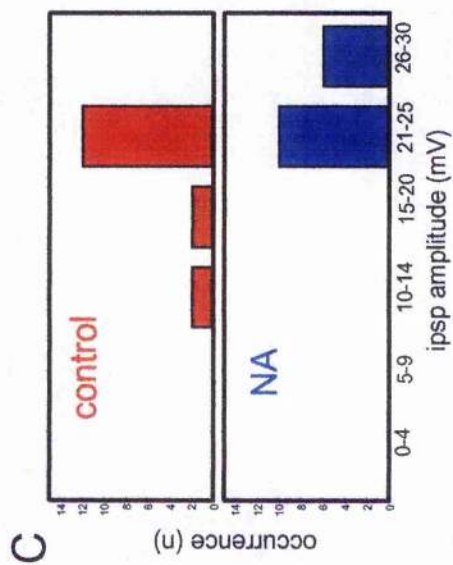
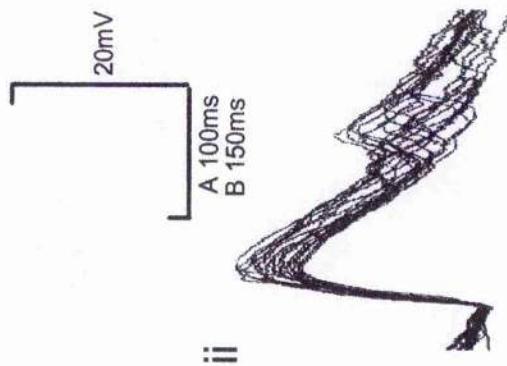
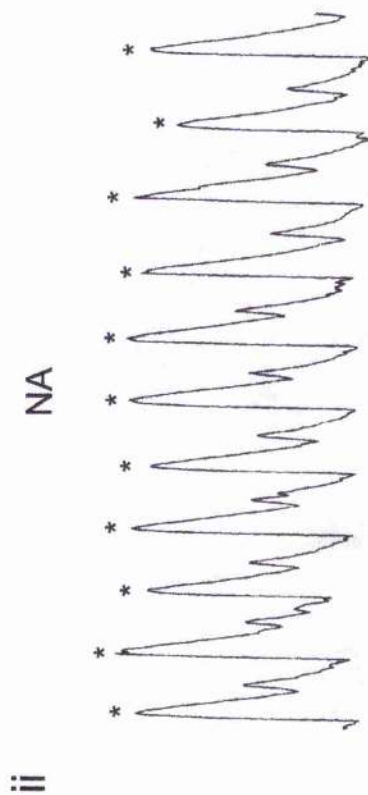
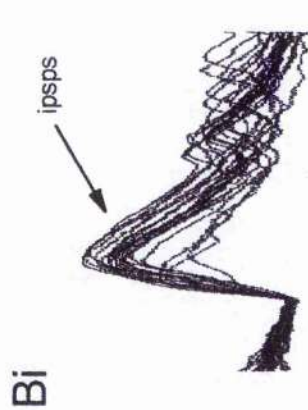
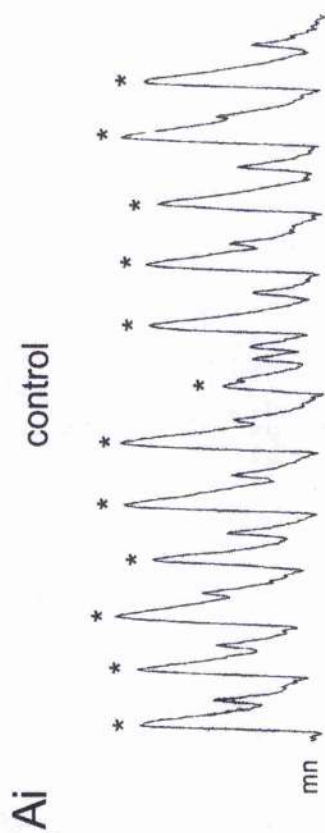
A. Exerpts of fictive swimming recorded from a motoneurone (mn) located at the level of the 4th post otic cleft taken from near the end of an episode of embryo swimming in control saline (i) and 8 minutes after the bath application of 2 μ M NA (ii). Note the enhancement in ipsp amplitude (asterisked in i,ii) after exposure to the amine.

B. Overlaps of 16 consecutive ipsps taken from near the end of the same episode as shown in A for both control (i) and NA (ii). Note the increase in size and decrease in variability of the ipsps in the presence of NA.

C. Amplitude distribution histograms for the same ipsps shown in B in both control (red bars) and eight minutes after addition of 2 μ M NA. Note that whilst in control saline, the measured ipsp amplitudes are between 10 and 25 mV, NA causes a shift in the amplitude distribution so that all potentials now lie between 21 and 30 mV. Mean amplitudes increased significantly ($p < 0.05$) from 20.9 ± 3.8 mV under control to 24.7 ± 2.2 mV eight minutes after the bath application of NA.

D. Distribution histograms of mean time to half-fall durations for the ipsps shown in B both in control (red bars) and after addition of NA (blue bars). Note that there are more ipsps of longer duration after exposure to NA. The mean time to half-fall for these ipsps significantly ($p < 0.05$) increases from 24.8 ± 1.9 ms in control to 26.8 ± 2.2 ms after exposure to NA.

E. Time to half-fall plotted against amplitude for ipsps ($n=100$) recorded during an episode of swimming from a motoneurone located at the level of the 12th post otic cleft of a stage 37/8 embryo. Recordings were made using KCl-filled electrodes. The correlation between amplitude and duration is highly significant ($p < 0.001$) with $r = 0.89$ and the equation of the fitted line is; time to half-fall = $0.89 + 1.04(\text{amplitude})$.



both the enhancement of ipsp amplitude and reduction of amplitude variability by NA more clearly. This shift in the distribution of ipsp amplitudes is illustrated in the histograms in figure 4.1C. Here it can be seen that NA increased the occurrence of higher amplitude ipsp (red bars) when compared to control (blue bars). As such, the mean ipsp amplitude increased significantly ($p < 0.05$) from 20.9 ± 3.8 mV in control saline to 24.7 ± 2.2 mV after exposure to NA. When the duration of the ipsp was examined for each experimental condition, they were also found to become longer in the presence of NA. Figure 4.1D shows the distribution of the time to half-fall values for the ipsp illustrated in 4.1A. It can be seen that after exposure to NA, a shift in the ipsp half-fall values occurred so that there was a greater number of longer duration potentials in the presence of NA. The amine-induced a significant ($p < 0.05$) change mean in half-fall time from 24.8 ± 1.9 ms in control to 26.8 ± 2.2 ms after exposure to NA. When the relationship between ipsp time to half-fall and duration is plotted, it was found that these two parameters were positively correlated so that larger ipsp had longer half-fall times. Figure 4.1E shows an example of this relationship in which ipsp time to half-fall is plotted against amplitude. The data in this panel are taken from another experiment in which recordings were made from a more caudal stage 37/8 embryo motoneurone (where ipsp tend to be more variable in amplitude -see below). It can be seen that the correlation between these two parameters of mid-cycle inhibition was strong ($r = 0.89$). Resting membrane potentials during the experiment shown in figure 4.1A-D were -68mV in control and -68mV after an 8 minute exposure to NA. Unfortunately the electrode blocked shortly after switching back to control saline so no wash was obtained for this experiment. However, in other experiments, the effects of NA were found to be reversible. These findings suggest that NA is capable of strengthening rostral inhibition in *Xenopus* embryos.

Since the amplitude of mid-cycle inhibition in the embryo spinal cord has been shown to decline in a rostrocaudal direction (Tunstall & Roberts, 1994; see introduction), I also examined the effects of the amine on caudal motoneurons where mid-cycle ipsp's have been reported to be smaller or absent altogether (Tunstall & Roberts, 1994). Preliminary results obtained from recordings of two caudal embryo motoneurons suggest that NA (concentrations used were 3 and 4 μ M) can also enhance glycinergic inhibition in this region of the spinal cord. For example, figure 4.2 shows an excerpt of activity recorded from an embryo motoneuron located at the level of the 14th post otic cleft in which mid-cycle ipsp's (asterisked in 4.2Ai) during swimming were, on average, much smaller and occurred far less reliably than those in rostral motoneurons (c.f. figure 4.1Ai). This finding is in partial agreement with Tunstall & Roberts (1994) who reported that the synaptic inhibitory drive to motoneurons during swimming weakens in a longitudinal direction. However, they found that caudal to the 12th post otic cleft, no ipsp's are present at all during swimming. In contrast to this report, the results presented here show clearly that ipsp's can occur on some cycles of locomotor activity in these caudally positioned motoneurons (asterisked in figure 4.2Ai). When NA was bath applied to caudal motoneurons, it caused a reversible enhancement in both the probability of occurrence and amplitude of the mid-cycle ipsp's. As can be seen from figure 4.2B, 3 μ M NA enhanced mid-cycle inhibition so that it was now of generally higher amplitude and apparent on virtually all cycles of swimming (asterisked in figure 4.2Aii). After 20 minutes wash, the probability of ipsp occurrence during swimming was again much lower. The overlaps of 16 consecutive cycles of activity taken from towards the end of an episode of swimming (figure 4.2B) clearly show the reversible NA-induced enhancement of the mid-cycle ipsp's. Figure 4.2C illustrates the effect histogramatically. It can be seen that whilst in control conditions, the large majority of measured cycles had either no or small amplitude ipsp's (red bars), a

Figure 4.2. NA enhances mid-cycle inhibition in caudal embryo motoneurones.

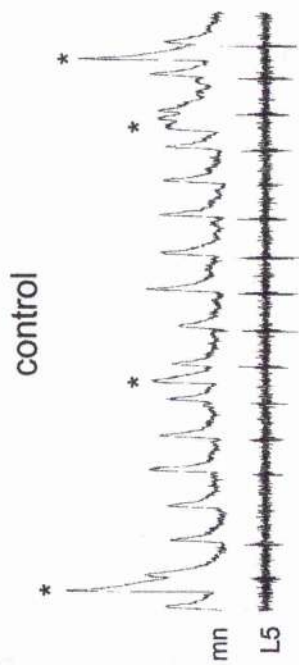
A. Exerpts of fictive swimming recorded from a motoneurone (mn) located at around the 14th post otic cleft and taken from near the end of an episode of stage 37/8 embryo swimming in control saline (i), 10 minutes after the bath application of $3\mu\text{M}$ NA (ii) and after a 20 minute wash in control saline (iii). Note that although there are occasional mid-cycle ipsp's in control (asterisked in i), they do not occur on every cycle of swimming. NA causes an increase in ipsp occurrence and amplitude (asterisked in ii), which is reversible upon wash (iii).

B. Overlaps of 16 consecutive ipsp's taken from near the end of the same episode as shown in A. NA causes an increased probability that motoneurones receive inhibition on each cycle of activity and also causes ipsp amplitudes to become larger and of more uniform amplitude.

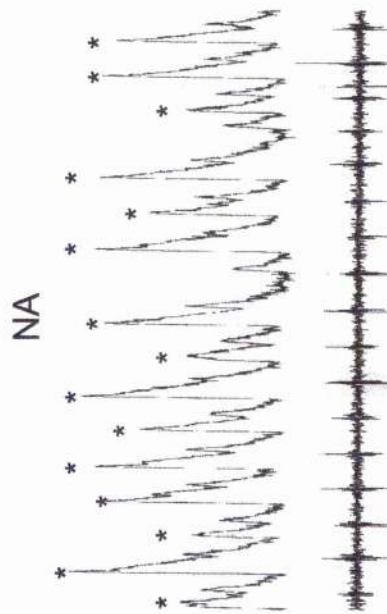
C. Amplitude distribution histograms for the same ipsp's shown in B. Note that in control saline (red bars), the majority of cycles have small or absent ipsp's. Further addition of NA (blue bars) causes the occurrence of ipsp's on nearly every cycle, with amplitudes ranging from 25 to 49 mV. A wash to control saline reverses this effect (green bars). When the mean of these ipsp's was calculated for each experimental condition, it was found that NA significantly ($p < 0.001$) increases mean ipsp amplitudes from 5.9 ± 12.8 mV in control conditions to 41.4 ± 6.6 mV 10 minutes after exposure to NA, an effect which is reversed to 7.8 ± 15.1 mV upon 20 minutes wash in control saline.

D. Time to half-fall distribution histograms of the ipsp's shown in B. Largely due to the absence of mid-cycle inhibition in control conditions (red bars), the majority of time to half-fall values fit into the 0-4 ms category. Addition of NA (blue bars) results in a shift in the time to half-fall distribution so that the majority of cycles have ipsp's ranging from 25 to 54 ms. This effect is reversed on wash (green bars). Mean half-fall times significantly ($p < 0.001$) increased from 6.0 ± 13.5 ms in control saline to 41.3 ± 6.8 ms after 10 minutes exposure to $3\mu\text{M}$ NA. A further 20 minute wash to control saline caused half-fall durations to significantly drop to 7.8 ± 15.1 ms.

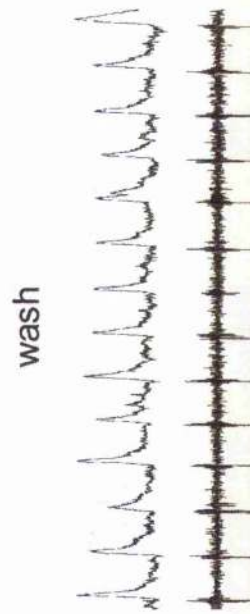
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ii



iii



Bi



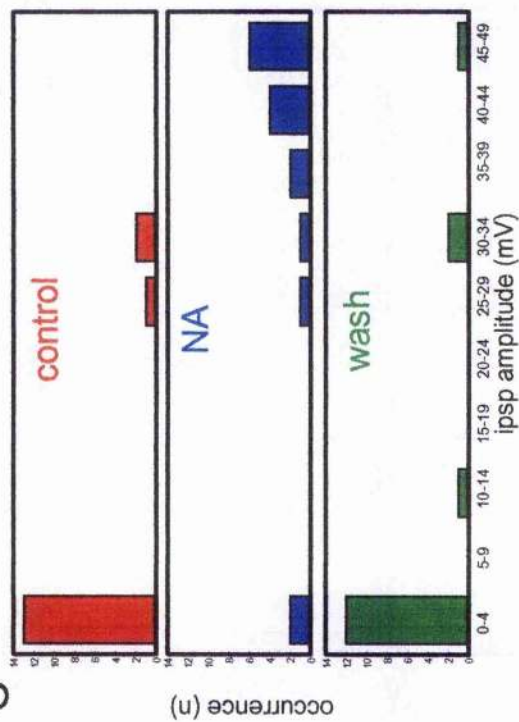
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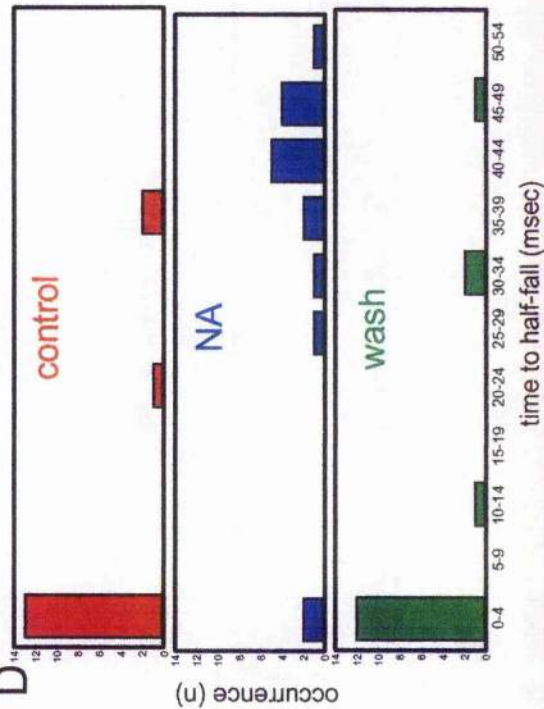
iii



C



D



10 minute exposure to NA ($3\mu\text{M}$ in this case) shifted the distribution so that relatively large amplitude ipsp's now occurred on nearly every cycle of activity (blue bars). A 20 minute wash to control saline reversed this effect (green bars). The mean amplitude of these ipsp's was found to significantly ($p < 0.001$) increase from 5.9 ± 12.8 mV in control saline to 41.4 ± 6.6 mV after exposure to NA. When the time to half-fall of the ipsp's was measured for these 16 overlaps (figure 4.2D), it was found that in control conditions, the majority of ipsp's amplitudes fell in the 0-4 ms category (red bars). This was largely due to the absence of ipsp's on most cycles within the episode. In contrast, exposure to NA caused a much greater incidence of ipsp's with time to half-fall durations ranging from 25-54 ms (blue bars). As a result, the mean half-fall times significantly increased from 6.0 ± 13.5 ms in control saline to 41.3 ± 6.8 ms after exposure to NA. The effects on both amplitude and duration were found to be reversible (figure 4.2Aiii, Biii, C, D). Resting membrane potentials during this experiment were -56 mV in control, -57 mV 10 minutes after addition of NA and -52 mV during wash.

In summary, the findings presented above show that NA affects the amplitude and duration of mid-cycle ipsp's during swimming in *Xenopus* embryos. This effect appears to be most prominent in caudal regions of the spinal cord (where inhibition is generally much less prominent) as opposed to rostral regions (where the inhibition is normally much stronger).

ii) NA enhances rostral mid-cycle inhibition in the stage 42 larvae.

The effects of NA ($1\text{-}20\mu\text{M}$) were also examined in rostral larval stage 42 motoneurons where mid-cycle ipsp's are more variable in amplitude and can drop out altogether on some cycles of activity, especially towards the end of an episode (see figure 4.3Ai). When NA was applied to rostrally located larval

motoneurons ($n=7$), it enhanced the amplitude and reduced the variability of mid-cycle ipsp during swimming. The example illustrated in figure 4.3 is made from a motoneurone located at the level of 5th post otic cleft of a larval preparation. 10 minutes after the bath application of $4\mu\text{M}$ NA, mid-cycle ipsp (asterisk in 4.4Aii) became much larger and less variable in amplitude. The overlaps of 16 consecutive ipsp taken from towards the end of the episode in figure 4.3B show this effect more clearly. This shift to higher ipsp amplitudes is illustrated in the histogram of figure 4.3C, where it can be seen that whilst there was a spread of ipsp amplitudes ranging from 0-34mV in control saline (red bars), ipsp of 25 mV or greater occurred on every cycle of activity in the presence of NA (blue bars). As such, the mean amplitude of these ipsp was found to significantly ($p<0.001$) increase from 18.8 ± 11.9 mV in control saline to 30.6 ± 1.8 mV after exposure to NA. A similar effect was also seen on the duration of these potentials so a greater number of ipsp had longer time to half-fall values after exposure to NA (Figure 4.3D). The mean time to half-fall duration was found to also significantly increase from 15.6 ± 12.2 ms in control saline to 20.8 ± 8.6 ms in the presence of NA. The effects on ipsp amplitude and duration were reversible upon return to control saline (figure 4.3Aiii,Biii,C,D). Resting membrane potentials during this experiment were -75 mV in control, -72mV after 10 minutes exposure to NA and -78mV after a 12 minute wash. The effect of NA on caudal motoneurons at stage 42 was not examined during the course of this study.

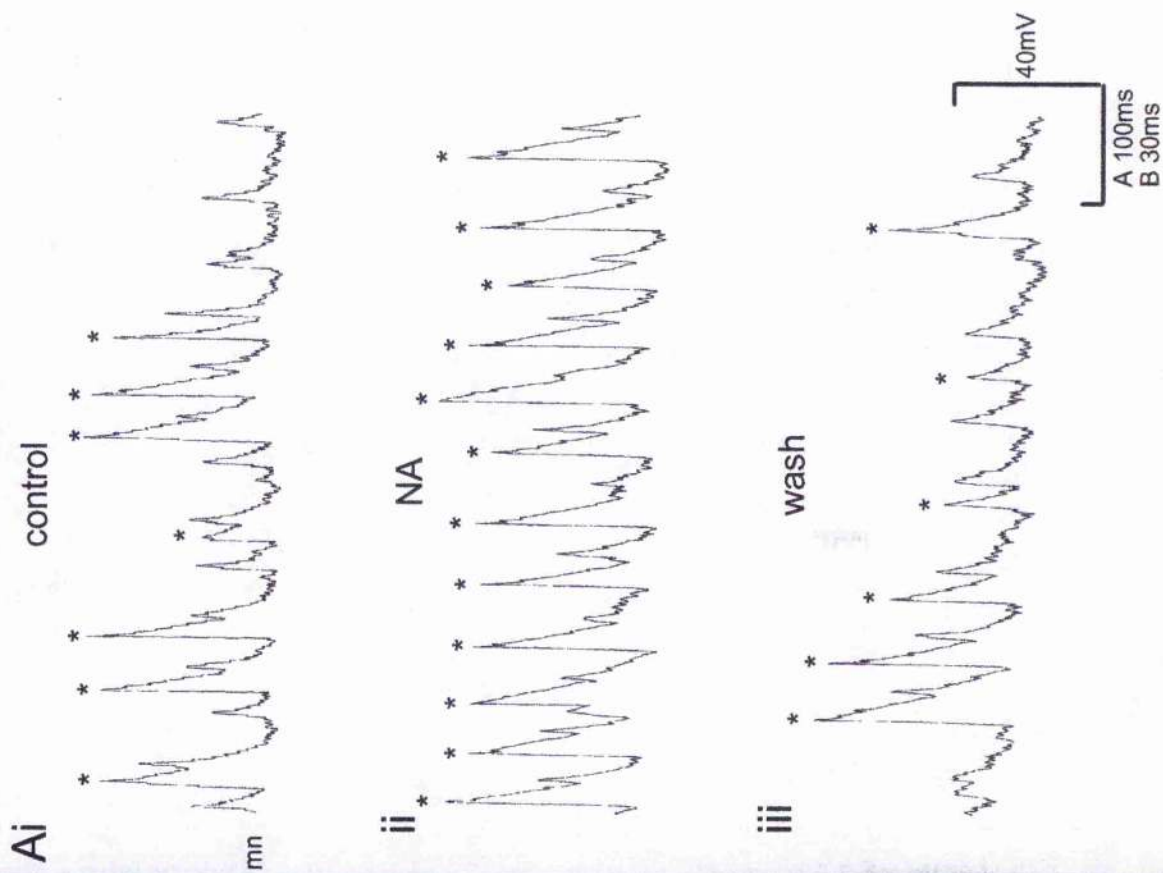
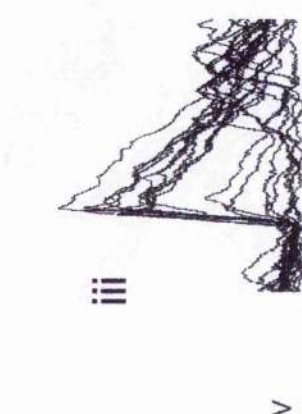
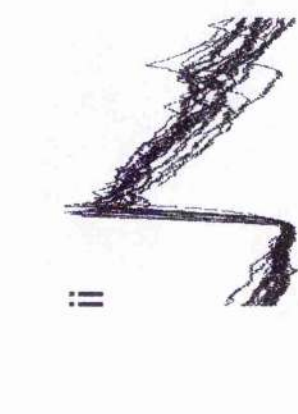
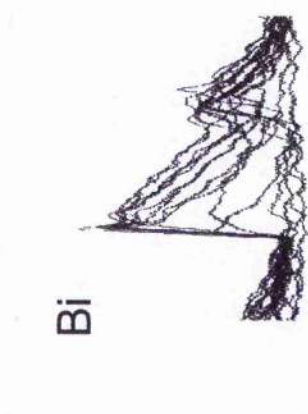
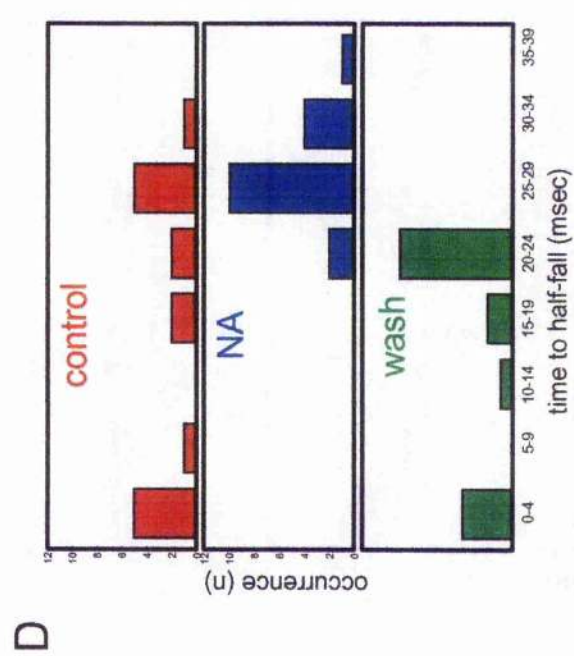
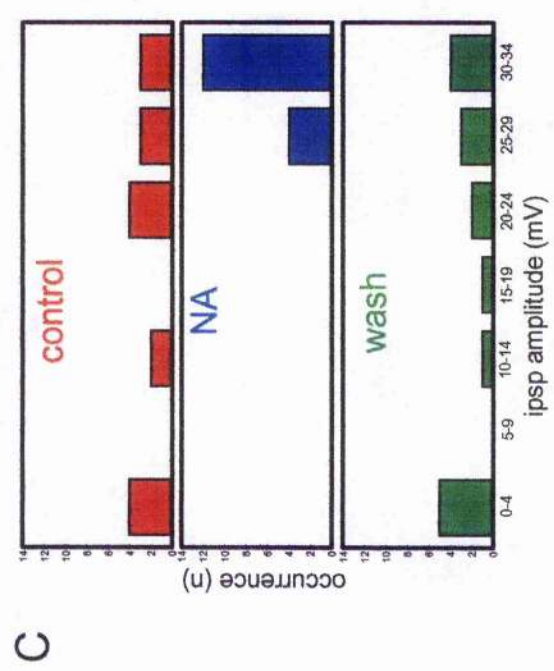
Figure 4.3. NA enhances mid-cycle inhibition in rostral larvae motoneurones.

A. Exerpts of fictive swimming recorded from a motoneurone (mn) located at the level of the 5th post otic cleft and taken from near the end of an episode of larval swimming in control saline (i), 10 minutes after the bath application of 4 μ M NA (ii) and after a 12 minute wash to control saline (iii). Note that rostral ipsp amplitudes in control saline (asterisked in i) are more variable than those of the embryo (see figure 4.1). NA enhances mid-cycle ipsp amplitude and occurrence (asterisked in ii), an effect that is reversible upon wash to control saline (iii).

B. Overlaps of 16 consecutive ipsp's taken from near the end of the same episode as shown in A. NA causes an increased probability that motoneurones will receive inhibition on each cycle of activity and also causes ipsp's to become larger and of more uniform amplitude.

C. Amplitude distribution histograms showing that whilst in control saline (red bars) a wide spread of ipsp amplitudes occurs, ranging from 0 to 34 mV, subsequent exposure to NA (blue bars) reduces the distribution of ipsp amplitudes so that all are of 25 to 34 mV in amplitude. A wash to control saline (green bars) reverses this effect. The mean increase in amplitude for these ipsp's is from 18.8 ± 11.9 mV under control to 30.6 ± 1.8 mV under NA. A 12 minute wash in control saline reversed the effects of NA, so that mean ipsp amplitude fell to 18.3 ± 13.5 mV. All effects are significant ($p < 0.001$).

D. The distribution of time to half-fall durations of the ipsp's shown in B shifts from a wide spread in control (red bars) to only longer duration potentials after exposure to NA (blue bars). This effect is reversible upon wash (green bars). The mean half-fall times of these ipsp's increases significantly ($p < 0.001$) from 15.6 ± 12.2 ms in control saline to 28.9 ± 1.0 ms after exposure to NA. A significant ($p < 0.001$) fall in duration to 20.8 ± 8.6 ms occurs after 12 minutes wash in control saline.



iii) Opposing effects of 5-HT and NA on mid-cycle inhibition in the larvae.

5-HT has already been reported to reduce the amplitude of and induce failures in mid-cycle ipsp's during swimming in *Xenopus* tadpoles (McDearmid, Scrymgeour-Wedderburn & Sillar, 1997), the opposite effect to that of NA. In some larval motoneurons where reciprocal inhibition was strong during control episodes of swimming, I examined the effect of NA on the weakened synaptic inhibition induced by prior exposure to 5-HT (n=3).

Bath application of 1-20 μ M 5-HT caused a reduction in the amplitude of mid-cycle inhibition during swimming. Figure 4.4 illustrates a typical example of this effect where in control saline, the mid-cycle ipsp's of this particular neurone, recorded from a stage 42 animal at around the 4th post-otic cleft were of reliably high amplitude even towards the end of an episode of swimming (asterisked in figure 4.4Ai). 10 minutes after the addition of 5 μ M 5-HT, at an equivalent point within the episode, the ipsp's were now found to wain and become more variable (asterisked in figure 4.4Aii). 10 minutes after subsequent addition of 2 μ M NA in the presence of 5-HT, mid-cycle ipsp's became consistently larger in amplitude (asterisked in figure 4.4Aiii). Washing-off the NA in the presence of 5-HT for 20 minutes caused an increased variability in ipsp amplitude (figure 4.4Aiv). The ipsp overlaps taken from 16 consecutive cycles of activity near the end of episodes of swimming under each condition in figure 4.4B show clearly that 5-HT increased the variability and reduced the amplitude of the ipsp's during swimming (Bii c.f. Bi). The addition of NA caused ipsp's to become less variable and larger in amplitude (Biii), an effect which is reversible on wash in 5-HT (Biv). 5-HT and NA therefore have opposite effects on ipsp amplitudes, as shown in the amplitude distribution histograms in figure 4.4C. The amines also had opposing effects on the distribution of half-fall times of the ipsp's (figure 4.4D). The resting membrane potentials during this experiment were -89mV in

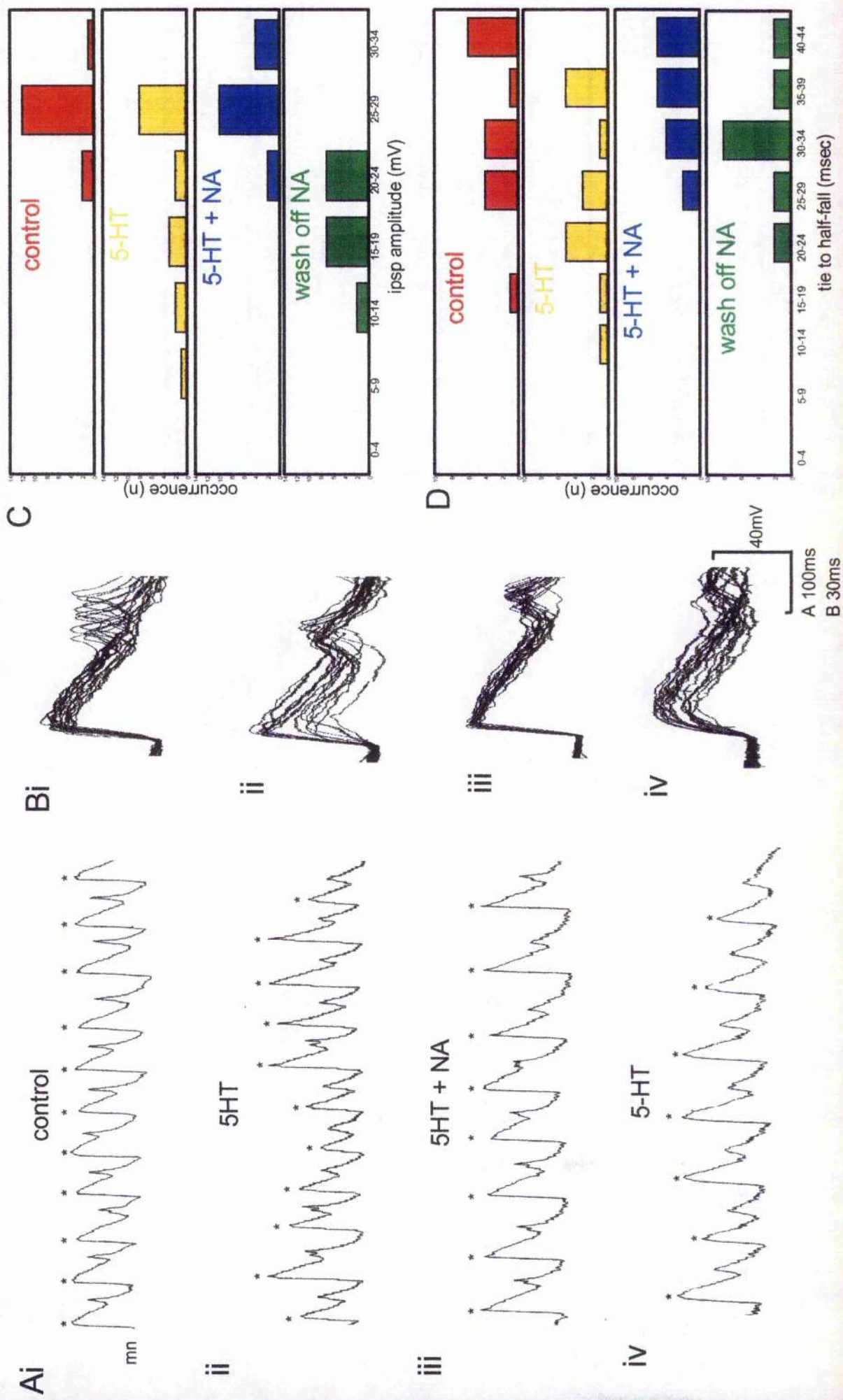
Figure 4.4. Effects of NA on 5-HT induced depression of mid-cycle inhibition.

A. Exerpts of fictive swimming recorded from a motoneurone (mn) located at the level of the 4th post otic cleft and taken from near the end of an episode of larval swimming in control saline (i), 10 minutes after the bath application of 5 μ M 5-HT (ii), after a 10 minute additional application of 2 μ M NA (iii) and after a 20 minute wash in 5 μ M 5-HT (iv). Note that 5-HT reduces ipsp amplitudes (ii) whereas subsequent addition of NA enhances them (iii).

B. Overlaps of 16 consecutive ipsp's taken from near the end of the same episode as shown in A. Control ipsp amplitudes are high (i). 5-HT reduces the amplitude of this component of the synaptic drive (ii), whereas 2 μ M NA reverses this effect (iii). A wash in 5 μ M 5-HT for 20 minutes causes the ipsp's to become more variable (iv).

C. Amplitude distribution histograms showing that whilst in control, ipsp amplitudes range between 20 and 34 mV, further addition of 5-HT (yellow bars) causes the distribution to shift so that there is now a spread of ipsp amplitudes from 0 to 34 mV. Subsequent addition of NA (blue bars) reverses this effect so that ipsp amplitudes only range from 20 to 34 mV. A wash in 5-HT (green bars) reduces the number of higher amplitude ipsp's. The mean amplitude for these ipsp's was also calculated. 5-HT decreased amplitudes (significant to $p < 0.02$) from 27.0 ± 2.3 mV in control to 21.4 ± 8.8 mV after a 10 minute exposure to the amine. Further addition of 2 μ M NA caused amplitudes to increase (significant to $p < 0.01$) to 27.9 ± 2.2 mV. A wash in 5 μ M 5-HT for 20 minutes causes ipsp amplitudes to fall to 19.4 ± 3.2 mV.

D. Time to half-fall distribution histograms of the ipsp's shown in B. In control saline (red bars), the majority of ipsp's range in duration from 25 to 44 ms. 5-HT (yellow bars) increases the number of shorter amplitude ipsp's. Further addition of NA (blue bars) reverses this effect so that all measured ipsp's now range between 24 and 44 mV. A wash in 5-HT again increases the incidence of lower amplitude ipsp's (green bars). Mean durations of these potentials decreases from 34.6 ± 7.6 ms in control saline to 27.6 ± 12.0 ms after exposure to 5-HT, an effect which is reversed by 2 μ M NA, with ipsp half-fall time increasing significantly ($p < 0.01$) to 35.7 ± 5.3 ms. A 20 minute wash in 5 μ M 5-HT decreases half-fall time to 32.5 ± 1.2 ms.



control saline, -92mV after 10 minutes exposure to 5-HT, -88mV after subsequent addition of NA and -92 mV after wash in 5-HT.

When 5-HT (1-30 μ M) was bath applied to animals that had already had their mid-cycle inhibition enhanced by 1-10 μ M NA, the opposite effect was found so that 5-HT reversed the effects of NA (n=3). In the example shown in figure 4.5, bath application of NA (4 μ M for 10 minutes in this case) enhanced mid-cycle inhibition (figure 4.5Aii, Bii) whilst further addition of 5-HT (3 μ M for 10 minutes in this case, figure 4.5Aiii, Biii) caused ipsp amplitudes to decline. Strychnine (1 μ M) was added at the end of this experiment to show that the ipsp were blocked by the glycine antagonist (Aiv, Biv). The histograms in figure 4.5C shows the effects on ipsp amplitude distributions for the same data depicted in B. Here it can be seen that whilst there was a wide spread of ipsp amplitudes in control saline (red bars), exposure to NA caused only higher amplitude ipsp to occur (blue bars). Further addition of 5-HT had the opposite effect so that there was now a greater spread in ipsp amplitudes. The changes in ipsp amplitudes induced by the two amines were also reflected in the mean time to half-fall shown in figure 4.5D, where it can be seen that whilst NA caused a greater incidence of longer duration ipsp, subsequent addition of 5-HT had the opposite effect, producing a higher number of shorter ipsp. Resting membrane potentials for this experiment were -82mV in control, -82 mV after exposure to NA, -85mV after further addition of 5-HT and -71mV after exposure to strychnine. The results indicate that the amplitude of ipsp during larval swimming may depend on the relative concentrations of 5-HT and NA present within the spinal cord.

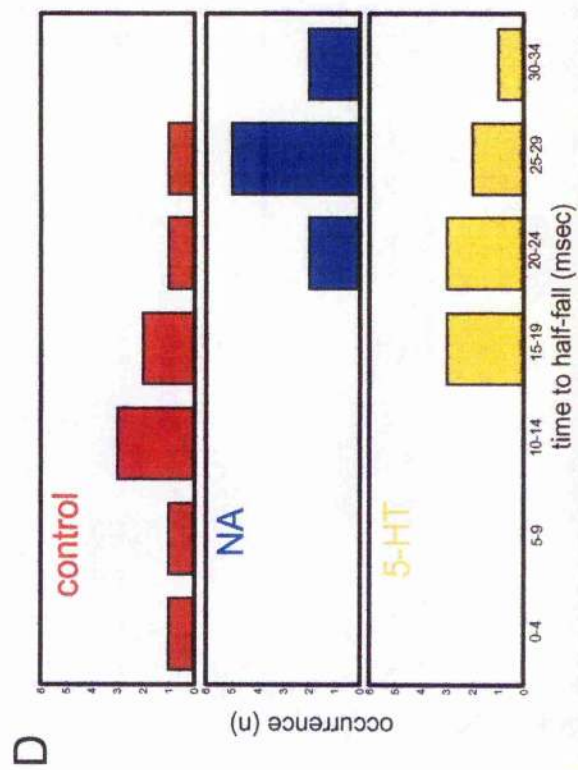
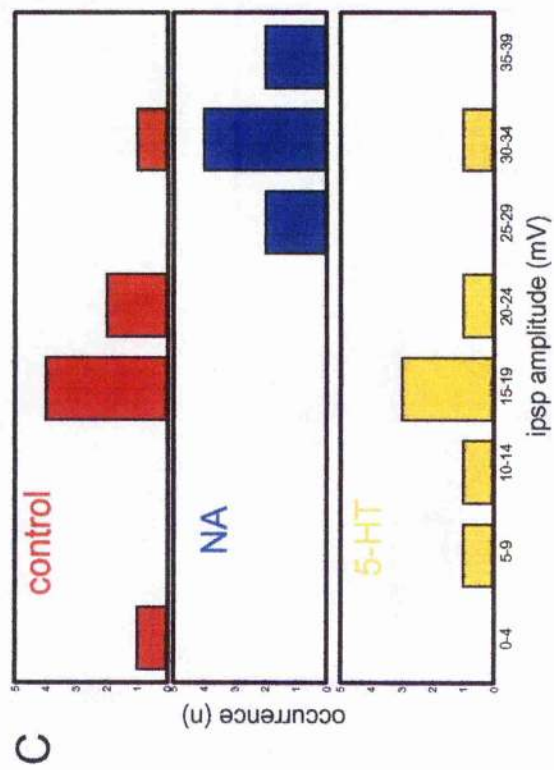
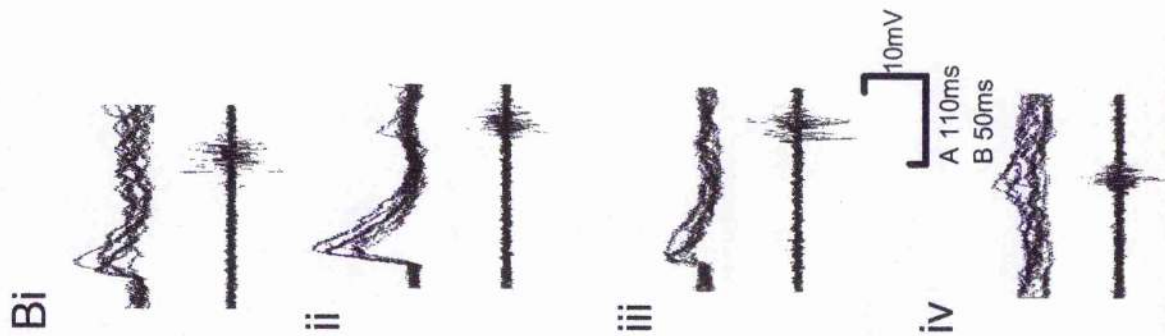
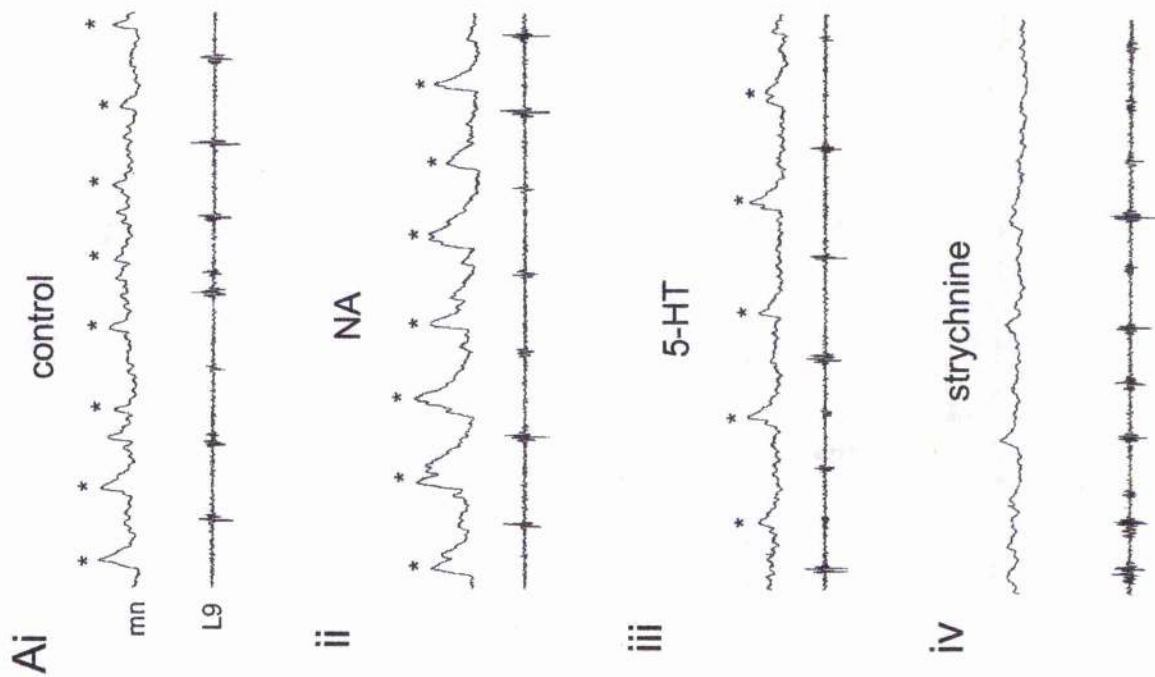
Figure 4.5. Effects of 5-HT on NA induced potentiation of mid-cycle inhibition.

A. Exerpts of fictive swimming recorded from a larval motoneurone (mn) located at the level of the 4th post otic cleft and the 9th post otic ventral root cleft taken from near the end of an episode of swimming in control (i), 10 minutes after the bath application of $4\mu\text{M}$ NA (ii), after a 10 minute additional application of $3\mu\text{M}$ 5-HT (iii) and after a 20 exposure to strychnine ($1\mu\text{M}$, iv). Note that NA enhances ipsp amplitudes (Aii) whereas subsequent addition of 5-HT depresses them (Aiii). The ipsps are abolished by $1\mu\text{M}$ strychnine (iv).

B. Overlaps of 16 consecutive ipsps taken from near the end of the same episode as shown in A. NA potentates the mid-cycle inhibitory synaptic drive to motoneurones (Bii), whereas 5-HT reverses this effect (Biii).

C. Amplitude distribution histograms showing that whilst a spread of ipsps amplitudes occurs in control saline (red bars), exposure to NA (blue bars) causes the occurrence of only higher 25 to 39 mV amplitude ipsps. Subsequent exposure to 5-HT (yellow bars) reverses this effect. The mean increase in amplitude for these ipsps was also calculated. $4\mu\text{M}$ NA increased mean amplitude from 17.4 ± 8.4 mV in control saline to 33.2 ± 7.3 mV after 10 minutes exposure to the amine. Further addition of 5-HT caused a significant ($p < 0.01$) drop in mean amplitudes to 19.0 ± 9.9 mV.

D. Time to half fall distributions for the same ipsps shown in B. The occurrence of longer duration potentials also increases after bath application of NA. Further addition of 5-HT has the opposite effect so that there is a wider spread of ipsp amplitudes. Mean amplitudes increased significantly ($p < 0.01$) from 15.4 ± 8.6 ms in control to 27.8 ± 3.8 ms after 10 minutes exposure to NA. Mean time to half-fall values significantly ($p < 0.01$) drop to 22.1 ± 5.1 ms after a 10 minute exposure to $3\mu\text{M}$ 5-HT in the presence of NA.



iv) NA facilitates the release of inhibitory transmitter.

Having established that NA is capable of enhancing the mid-cycle ipsp during fictive swimming, the mechanism(s) through which the amine may modulate inhibitory transmission was investigated. There are several possible ways in which NA could increase the amplitude of mid-cycle ipsp during swimming. Presynaptic mechanisms of action of NA could involve: i) an enhancement in the probability that commissural interneurons fire action potentials (thereby increasing the proportion of inhibitory interneurons that are normally active during swimming); ii) an increase in the probability of vesicular release from terminals of commissural interneurons; or iii) an increase in the quantal size so that more glycine is liberated into the synaptic cleft by each vesicle. Alternatively, NA could act postsynaptically to affect the probability of channel opening, alter the duration of channel opening, affect conductance properties, input resistance or time constants of the glycine receptor when the ligand is bound.

In order to investigate pre- versus postsynaptic mechanisms of action of NA, spontaneous (s) ipsp occurring in motoneurons were examined. In the absence of swimming, motoneurons always receive apparently spontaneous ipsp which become strongly depolarising when KCl is used as the electrolyte. As illustrated in figure 4.6A, these potentials fit into two distinct populations, distinguishable by their duration and pharmacology (figure adapted from Reith & Sillar, 1997). In one population the potentials last around 90-200ms and are blocked by the GABA_A receptor antagonist bicuculline, and so are presumably GABAergic in origin. The second population (that are much more frequent than the GABAergic potentials (Reith & Sillar, 1997)) have durations of around 20-80ms and are blocked by strychnine, suggesting that they are glycine receptor-

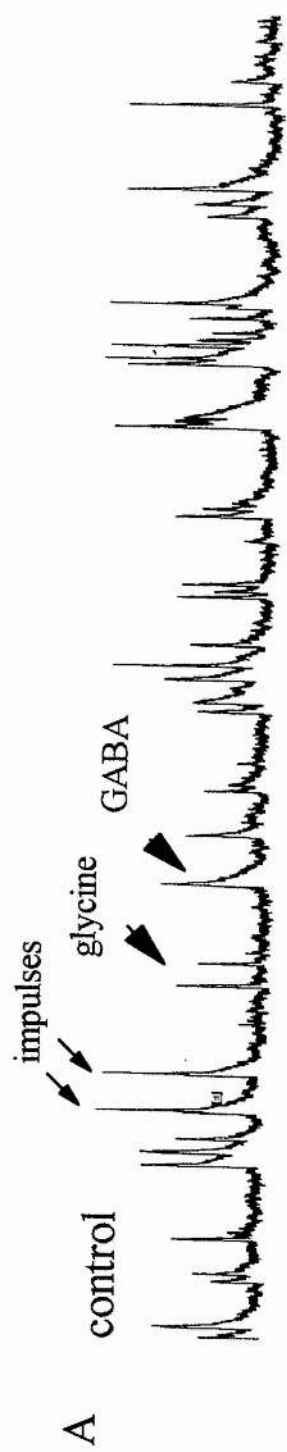
Figure 4.6 TTX-resistant spontaneous ipsp's.

A. Inhibitory potentials recorded in the absence of rhythmic activity resulting from the activation of both glycine and GABA_A receptors.

B. Bath application of TTX (0.5 μ M) to block sodium dependent spikes results in both GABA and glycinergic potentials remaining but a decrease in the number of each type of potential occurs.

C. Six overlapped traces of both glycine and GABA potentials recorded before and after exposure to TTX. Note the difference in duration of the potentials.

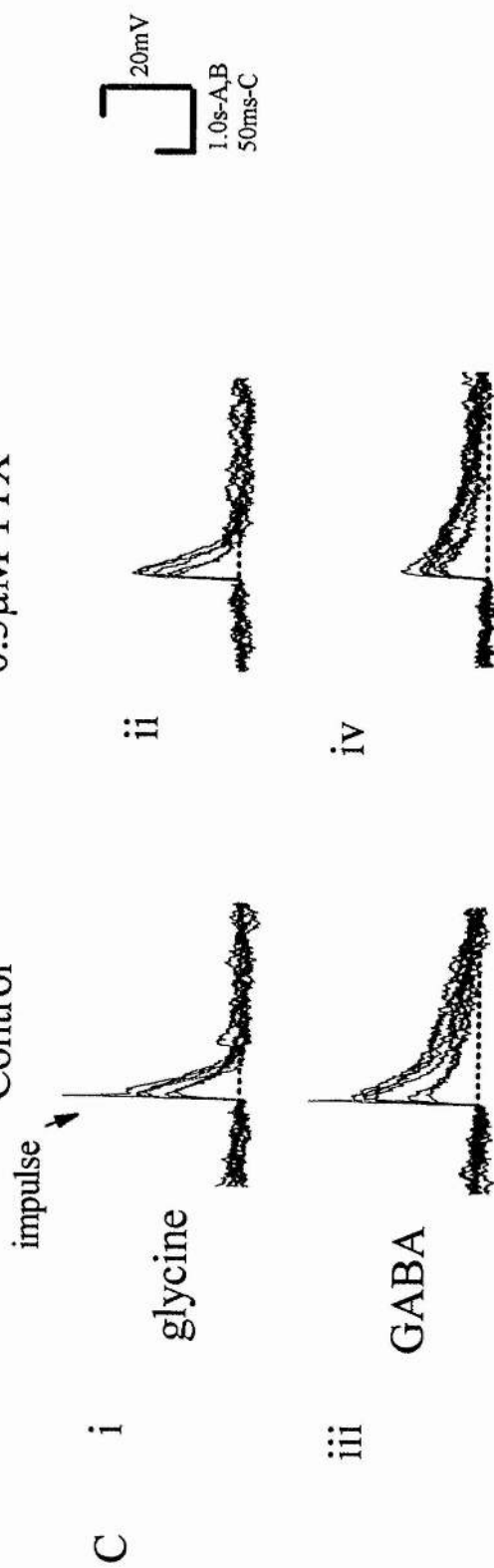
Figure adapted from Reith and Sillar (1997).



B 0.5 μ M TTX

0.5 μ M TTX

Control



mediated. The amplitudes of the inhibitory potentials in both populations fall into discrete size categories, presumably because they represent the quantal release of transmitter from GABAergic and glycinergic inhibitory interneurons. Following bath application of the sodium channel blocker tetrodotoxin (TTX) to abolish spike evoked transmitter release, the glycinergic and GABAergic ipsp's remain although the frequency of occurrence of these potentials is normally somewhat reduced (figure 4.6B). This suggests that although some of these ipsp's arise from sodium-spike evoked release of transmitter, a significant proportion of the potentials are due to spontaneous transmitter release from presynaptic terminals. The occurrence of glycinergic (s)ipsp's allowed a more direct investigation into how NA acts to potentiate glycinergic mid-cycle inhibition; a change in frequency of occurrence of glycinergic (s)ipsp's would presumably reflect a change in the probability of glycine release from commissural interneurons. A change in the amplitude distribution of the (s)ipsp's may reflect a change in the postsynaptic response to glycine or a change in the quantal content presynaptically.

To study the effect of NA on glycinergic transmission, recordings were made from motoneurons penetrated with KCl filled microelectrodes and the effect of NA (1-20 μ M) on the frequency and amplitude of the (s)ipsp's in the presence of TTX was examined. An example of the effect of NA on (s)ipsp release rate is shown in figure 4.7A. In the presence of 0.5 μ M TTX the frequency and amplitude distribution of glycinergic (s)ipsp's was measured during control conditions and 10 minutes after the application of 5 μ M NA. The amine reversibly increased the frequency of occurrence of glycinergic (s)ipsp's (n=6) so that in the example illustrated in figure 4.7 (s)ipsp rate significantly ($p < 0.001$) increased from 0.87 ± 0.09 Hz under TTX to 2.85 ± 0.45 Hz after addition of NA. Washing off the NA for 20 minutes reversed this effect (figure 4.7B). As these experiments were carried out in the absence of bicuculline, the frequency of occurrence of

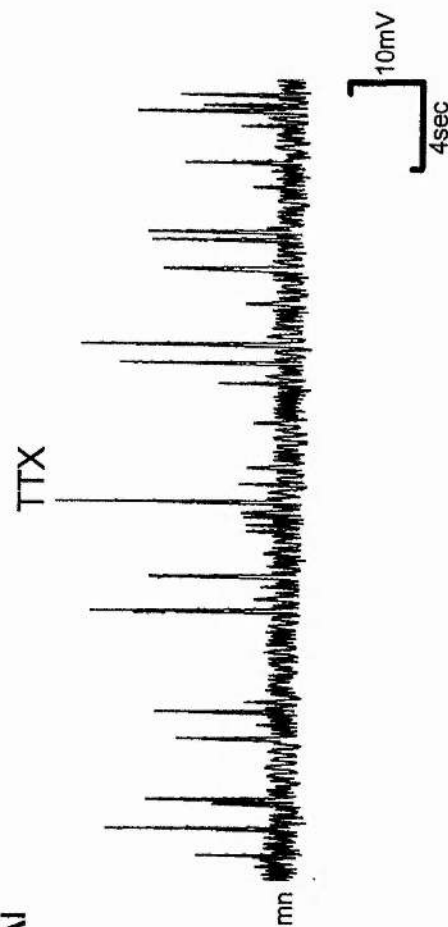
Figure 4.7. NA increases spontaneous ipsp rate.

A. Exerpts of activity recorded using a KCl-filled electrode from a presumed motoneurone at around the level of the 5th post otic cleft of a stage 42 larva in the presence of $0.5\mu\text{M}$ TTX. Depolarising spontaneous (s) ipsps occur under control conditions (i). A 10 minute exposure to $5\mu\text{M}$ NA increases the frequency of occurrence of these (s)ipsps (ii), an effect which is reversed upon 20 minute wash in control saline (iii).

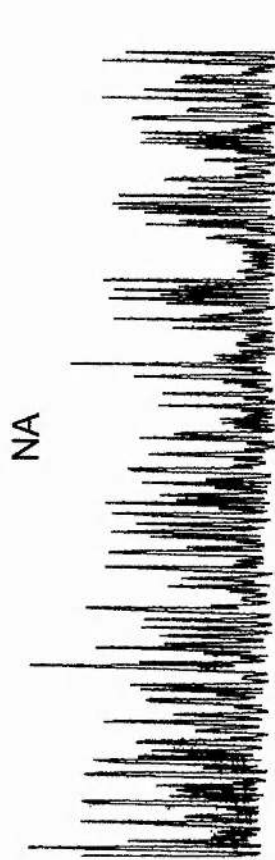
B. Histogram depicting mean frequency of glycinergic (s)ipsps, identified on the basis of their duration (see figure 4.6), under each experimental condition (frequencies averaged over a 3 minute period). $5\mu\text{M}$ NA increases (s)ipsp rate significantly ($p < 0.001$) from 0.87 ± 0.19 Hz under TTX to 2.85 ± 0.45 Hz after 10 minutes exposure to the amine. A 20 minute wash to control saline (with $0.5\mu\text{M}$ TTX added) decreases (s)ipsp rate to 2.0 ± 0.12 Hz.

C. Histogram depicting mean frequency of longer duration GABAergic (s)ipsps under each experimental condition (frequencies averaged over a 3 minute period). Frequency of GABAergic potentials significantly ($p < 0.001$) increases from 0.03 ± 0.01 Hz in $0.5\mu\text{M}$ TTX to 0.33 ± 0.06 Hz 10 minutes after bath application of $5\mu\text{M}$ NA, whilst a wash for 20 minutes in control saline (with $0.5\mu\text{M}$ TTX) caused GABAergic (s)ipsp frequency to drop to 0.14 ± 0.05 Hz. Data in A, B and C taken from the same experiment.

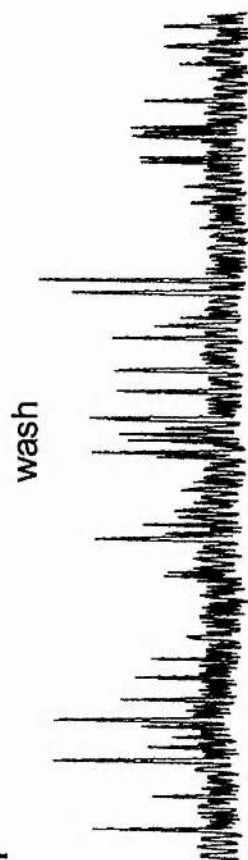
Ai



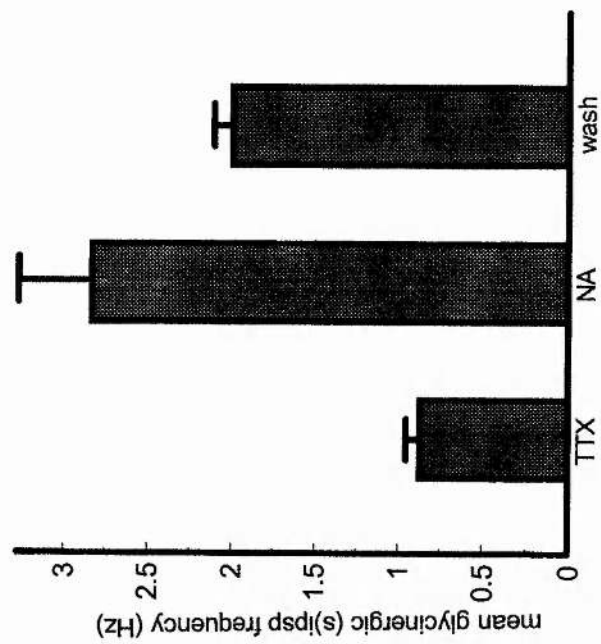
ii



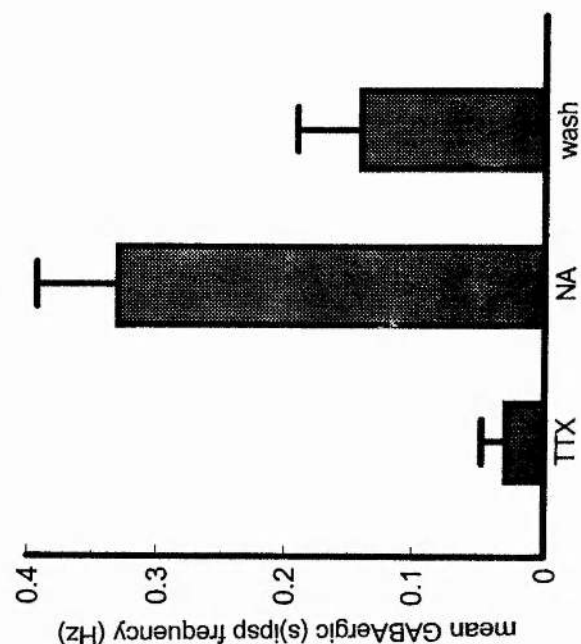
iii



B



C



GABAergic (s)ipsp, identified on the basis of their longer durations, was also examined. There was a similar effect on the frequency of these potentials after exposure to NA. Figure 4.7C illustrates this effect, where it can be seen that the frequency of spontaneous GABAergic potentials significantly ($p < 0.001$) increases after bath application of NA. This effect on the frequency of GABAergic potentials is reversed after 20 minutes wash in control saline (figure 4.7C). Resting membrane potentials in this experiment were -86mV under TTX, -84mV under NA and -88mV in wash. The amplitude distribution and duration of the glycinergic sipsps was found to be largely unaffected by NA (figure 4.8), suggesting a lack of postsynaptic effect of the amine. It was not possible to assess the effect of NA on the distribution of GABAergic ipsp amplitude as the potentials were so infrequent in control conditions.

These results indicate that NA can profoundly enhance the probability of both glycine and GABA release from the terminals of inhibitory interneurons. Furthermore, in the case of glycine at least, the amplitude distributions do not appear to be greatly affected implying that neither the quantal content nor the post-synaptic response to glycine are affected by NA.

v) Effect of NA on larval swimming frequency in the presence of glycine receptor antagonism.

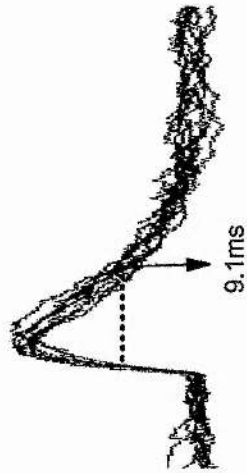
The preceding data suggests that NA can profoundly strengthen glycinergic inhibition during swimming via presynaptic facilitation of the glycine release mechanism. In chapter 3, I demonstrated that the principal effects of NA on the motor pattern were a lengthening of cycle periods and a decrease in rostrocaudal delays. The question arises as to whether these effects on the motor

Figure 4.8. Lack of effect of NA on amplitude distribution and duration of glycinergic (s)ipsp.

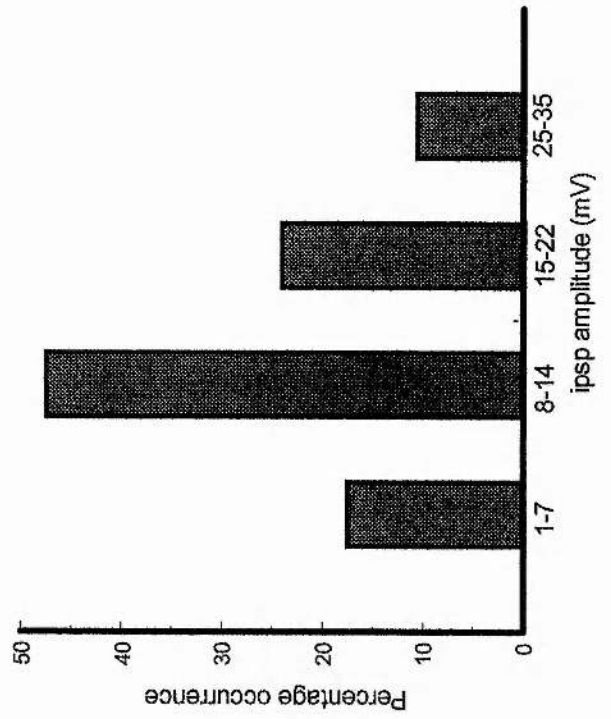
A. NA does not obviously affect the duration of the glycinergic (s)ipsp. Overlaps of 8 amplitude-matched (s)ipsp in TTX (Ai) and after the bath application of 5 μ M NA (Aii). The average duration of the potentials is 9.1 ms in TTX alone (i) and 9.2 after exposure to NA (ii).

B. Histograms showing amplitude distributions of (s)ipsp for TTX (Bi) and after the bath application of 5 μ M NA (Bii). The amplitude distributions are similar under each experimental condition.

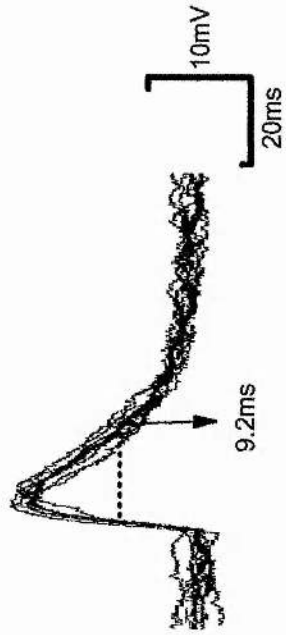
Ai



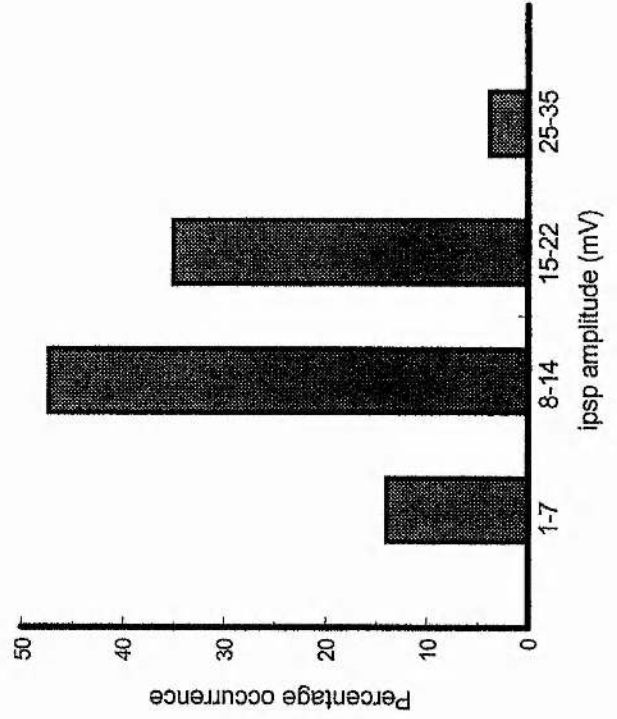
Bi



Aii



Bii



pattern can be accounted for by the amines modulation of glycinergic transmission.

If the NA-mediated effect on swimming frequency reported in chapter 3 is produced by a change in the strength of reciprocal glycinergic inhibition then the amine should not be able to affect motor output in the presence of strychnine. A series of experiments were therefore performed on stage 42 *Xenopus* tadpoles where the effects of strychnine on the ventral root activity underlying fictive swimming were examined in the presence of NA. Strychnine (1 μ M) was found to substantially reverse the effects of NA (1-10 μ M) on cycle period (n=9). A typical example of the effects on swim frequency are shown in figure 4.9. In this experiment the average cycle period under control conditions was 52.8 ± 2.1 ms. 10 minutes after bath application of 4 μ M NA, mean cycle period significantly ($p < 0.001$) increased to 68.0 ± 3.9 ms whilst addition of 1 μ M strychnine resulted in cycle periods significantly ($p < 0.001$) decreasing to 53.6 ± 3.8 ms so that they were no longer significantly different from control values ($p > 0.05$). In pooled data from five similar experiments, cycle periods were on average 36.9 ± 7.2 % greater than in control conditions after exposure to NA alone. Subsequent addition of strychnine caused swimming frequency to decrease so that average cycle periods were only 14.6 ± 7.7 % greater than control.

When strychnine was applied to block glycinergic transmission in *Xenopus* larvae before the addition of NA, similar results were obtained in that NA's ability to decrease swimming frequency was greatly reduced in the presence of strychnine (n=8). The example in figure 4.10 shows that whilst control cycle periods were on average 58.8 ± 7.9 ms, the addition of 1 μ M strychnine significantly ($p < 0.001$) decreased cycle periods to 44.0 ± 5.7 ms. This strychnine-induced acceleration in the motor pattern was in keeping with previous reports in both the embryo and the larvae (Roberts, Dale, Evoy & Soffe; 1985; Dale, 1995b; McDearmid, Sillar & Wedderburn, 1997). Subsequent addition of 5 μ M NA in the

Figure 4.9. Strychnine substantially reverses the increase in cycle periods mediated by NA.

A. Exerpts of fictive swimming activity recorded from L6 of a stage 42 larvae in control saline (i), 10 minutes after the bath application of 4 μ M NA (ii), and after a subsequent 15 minute exposure to 1 μ M strychnine (iii).

B. Histogram depicting cycle periods averaged from 30 cycles of activity taken from near the start of 3 separate episodes of swimming under each experimental condition. 4 μ M NA significantly ($p < 0.001$) increases cycle period from 52.8 ± 2.1 ms to 68.0 ± 3.9 ms. Subsequent Addition of 1 μ M strychnine resulted in cycle periods significantly ($p < 0.001$) decreasing to 53.6 ± 3.8 ms, so that they were no longer significantly different from controls ($p > 0.05$). Data in A and B taken from same experiment.

Ai

control



ii

NA



iii

NA + strychnine



B

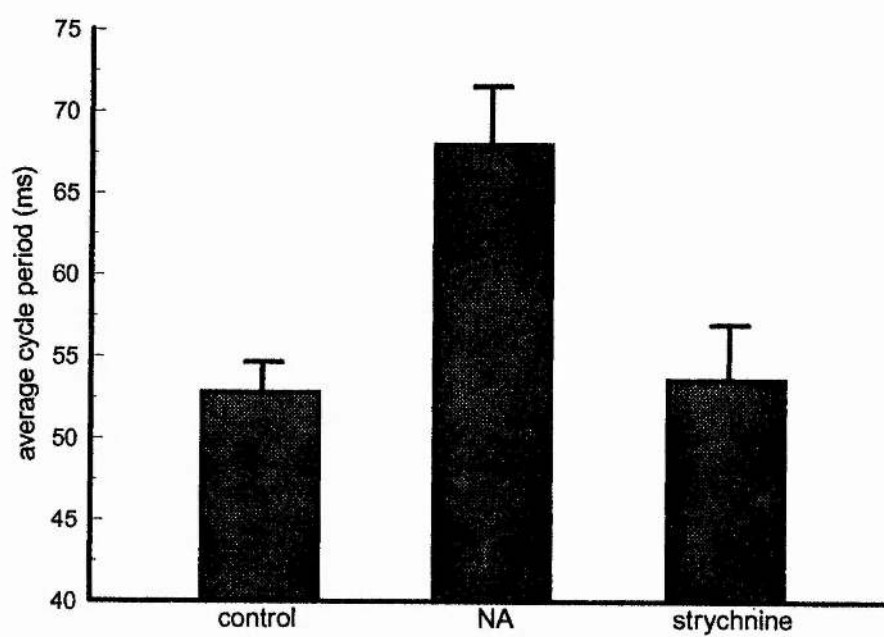


Figure 4.10. Effects of NA on cycle periods in the presence of strychnine.

A. Exerpts of fictive swimming activity recorded from L7 in a stage 42 larvae in control saline (i), 10 minutes after the bath application of $1\mu\text{M}$ strychnine (ii), and after a subsequent 10 minute exposure to $5\mu\text{M}$ NA (iii).

B. Histogram depicting average cycle periods under each experimental condition. From these results it can be seen that whilst strychnine significantly decreases average cycle periods ($p < 0.001$) from 58.8 ± 7.9 ms to 44.0 ± 5.7 ms, further application of NA only caused cycle periods to increase to 49.2 ± 3.8 ms.

Ai

control



ii

strychnine

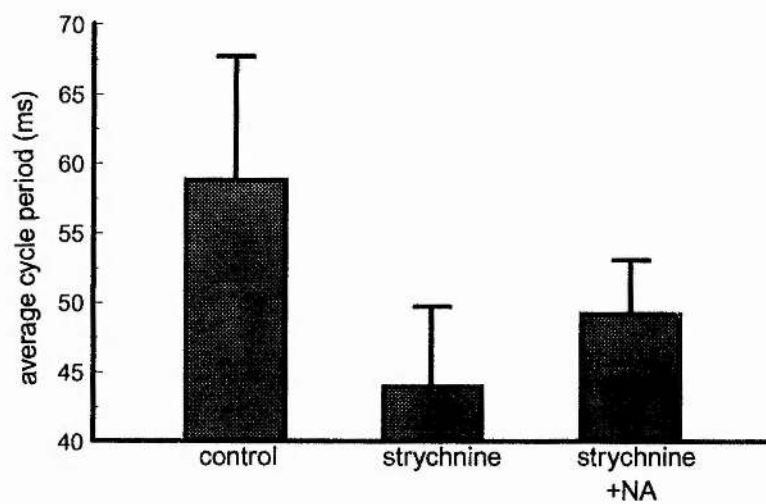


iii

strychnine + NA



B



presence of strychnine did significantly ($p < 0.001$) increase cycle periods, but only to 49.2 ± 3.8 ms. Therefore, although NA is capable of slowing the motor pattern to some extent in the absence of glycinergic inhibition, the amines potency appears to be much reduced. In pooled data taken from 6 different experiments, cycle period increased by only 9.9 ± 16.9 % when NA was applied in the presence of strychnine. This is in contrast to the average increase in cycle period of 34.8 ± 7.9 % when NA was applied alone. These data suggest that NA has a much less marked effect on cycle periods in the presence of strychnine, and so the enhancement of reciprocal inhibition during swimming may be largely responsible for the effects of NA on locomotor frequency.

vi) Effect of NA on longitudinal co-ordination during larval swimming in the presence of glycine receptor antagonism.

As discussed in chapter 3, a linear relationship exists between longitudinal delay and cycle period in stage 42 *Xenopus* larvae (Tunstall & Roberts, 1991; Tunstall & Sillar, 1993) so that as swimming frequency declines, longitudinal delays increase. This scaling of delays with cycle periods presumably helps the larva to maintain an optimum body shape for swimming over a wide range of frequencies. In chapter 3, I demonstrated that NA has a dramatic influence on longitudinal co-ordination so that in the presence of the amine, rostrocaudal delays are much smaller for any given cycle period. Could the NA-mediated decrease in this parameter of swimming also be dependent on an enhancement in reciprocal inhibition? In an attempt to answer this question, the effects of NA on the relationship between delay and cycle period was examined in the presence of strychnine. When strychnine ($1\mu\text{M}$) was applied to animals already exposed to NA ($1\text{-}10\mu\text{M}$), the amine-induced reduction in delays was largely reversed ($n=9$).

The excerpts of activity in figure 4.11A have been frequency-matched to show this effect more clearly and the graph in 4.11B shows that NA both increases cycle period and reduces longitudinal delays. Subsequent addition of 1 μ M strychnine is capable of substantially reversing the effects on delays, although it should be noted that it does not cause a full reversal. When delays are averaged for each experimental condition (figure 4.11C) this parameter of swimming significantly decreases ($p < 0.001$) from 24.8 ± 4.6 ms in control to 20.6 ± 4.6 ms after exposure to 5 μ M NA. Further addition of 1 μ M strychnine significantly ($p < 0.001$) increases mean delays to 22.8 ± 3.9 ms so that delays become closer to, although still significantly different ($p < 0.001$) from, those recorded in control conditions.

A similar result was obtained in preparations that were pre-treated with strychnine prior to the application of NA ($n=8$). Under these conditions NA's effects on longitudinal co-ordination were much less potent. Bath application of strychnine was found to increase longitudinal delays at any given frequency (see figure 4.12Aii,B). Subsequent addition of NA under these conditions causes delays to decrease to similar durations as those seen in control. The mean effect on delays for the same data presented in B is shown in figure 4.12C. Here it can be seen that strychnine causes a significant increase ($p < 0.001$) in delays from 17.4 ± 2.5 ms in control to 21.2 ± 2.2 ms after the bath application of 1 μ M strychnine. Although subsequent addition of 5 μ M NA causes delays to significantly ($p < 0.001$) decrease to 16.4 ± 2.0 ms, the observed increase is smaller than would be seen when NA is bath applied alone (see figure 3.10 in previous chapter). Hence the ability of NA to reduce longitudinal delays may be partly dependent on the presence of functional glycinergic inhibition. Nevertheless, the ability of NA to decrease delays in the presence of strychnine suggests that additional mechanisms are involved.

Figure 4.11. The effects of NA on longitudinal co-ordination are reduced by strychnine.

A. Frequency matched excerpts of stage 42 larval fictive swimming activity recorded from L3 and L13 in control (i), 10 minutes after the bath application of $5\mu\text{M}$ NA (ii) and after a 10 minute exposure to strychnine ($1\mu\text{M}$, iii).

B. Graph plotting longitudinal delay against cycle period for each consecutive cycle of activity in control saline ($n=214$), 10 minutes after addition of $5\mu\text{M}$ NA ($n=138$) and following a further 10 minutes exposure to $1\mu\text{M}$ strychnine ($n=106$). In control conditions, the correlation between delay and cycle period is highly significant ($p<0.001$) with the r value being 0.91. After 10 minutes exposure to $5\mu\text{M}$ NA, the correlation between delay and cycle period remains significant ($p<0.001$) with $r=0.82$, but the amine causes a shift in the relationship between delay and cycle period so that even though cycle periods increase, delays decrease. Subsequent addition of strychnine increases delays and decreases cycle period, causing a reversal of the NA effect. Although the correlation between delay and cycle period is still highly significant ($p<0.001$) in the presence of strychnine The r value after exposure to strychnine dropped to 0.62.

C. Histogram representing the mean delays for an experiment where $5\mu\text{M}$ NA was added prior to bath application of $1\mu\text{M}$ strychnine. NA causes mean delays to significantly ($p<0.001$) decrease from 24.8 ± 4.6 ms in control saline to 20.6 ± 4.6 ms whilst delays significantly ($p<0.001$) increased to 22.8 ± 3.9 ms after exposure to strychnine.

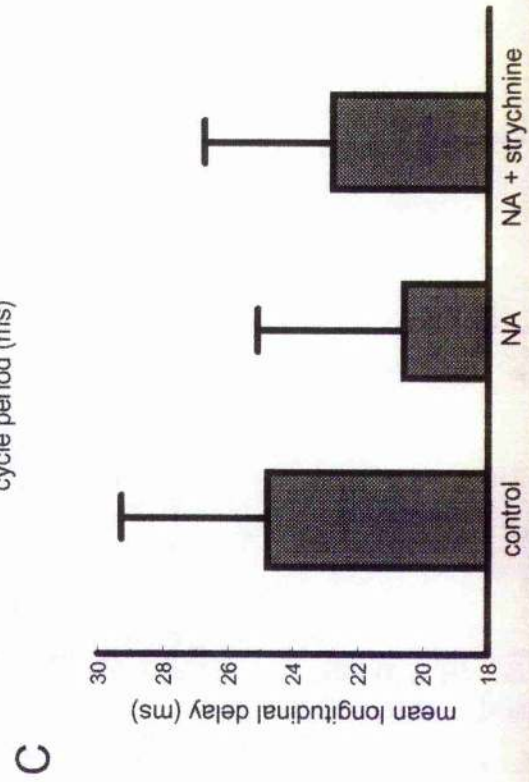
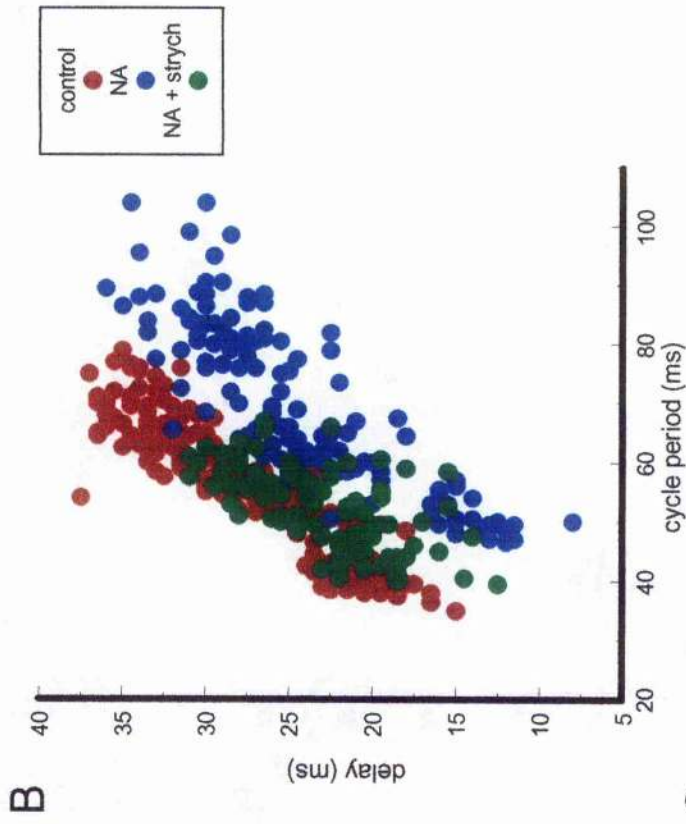
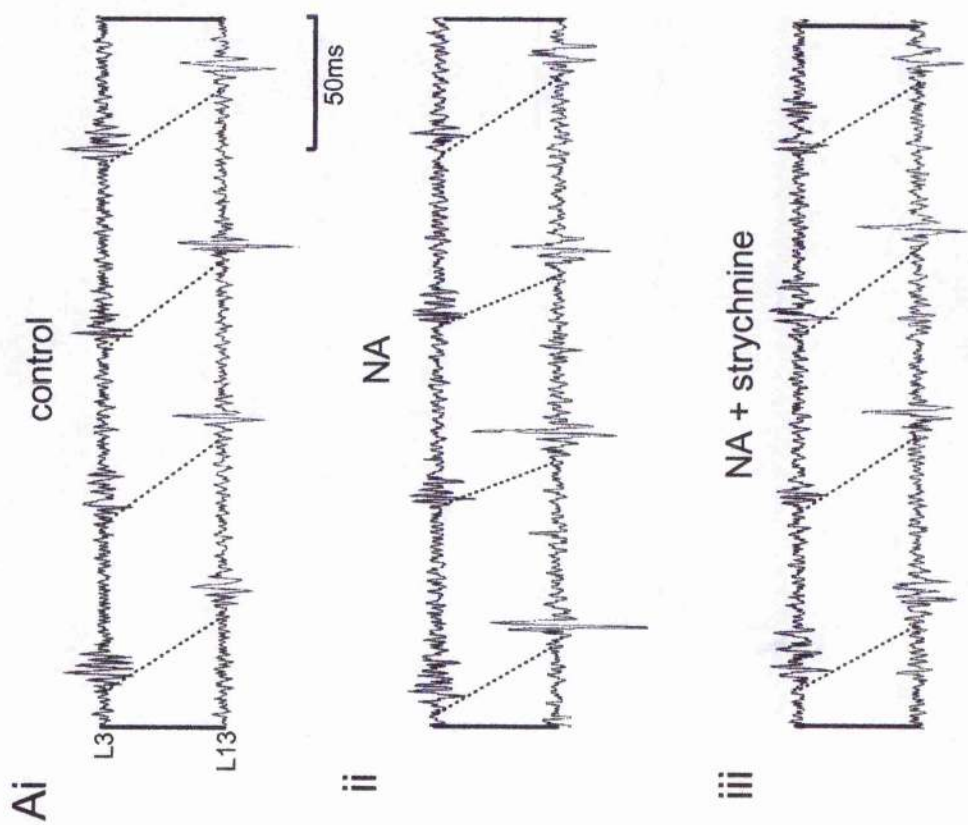
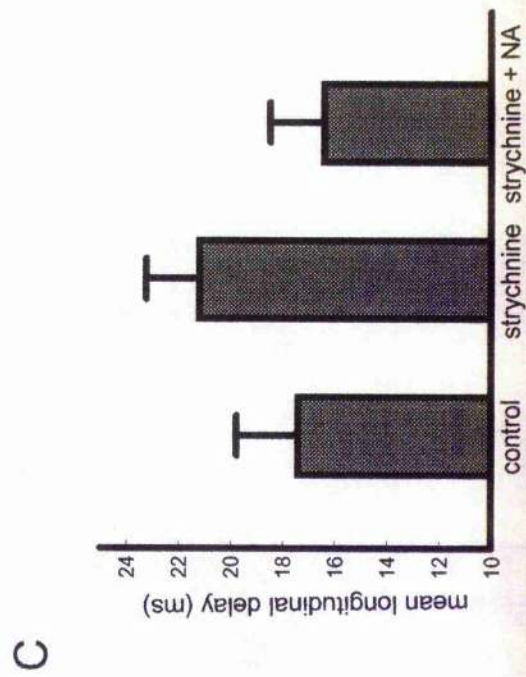
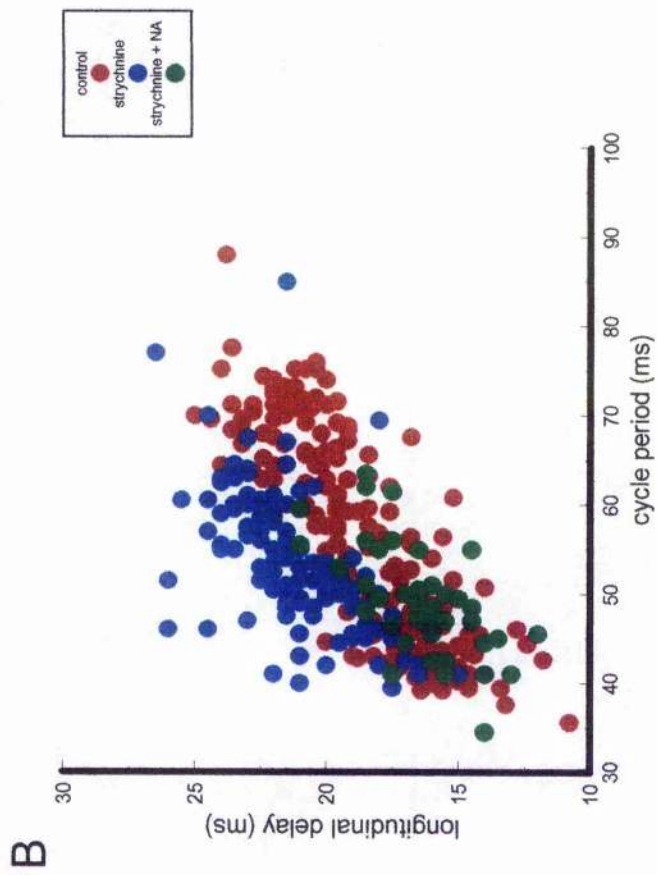
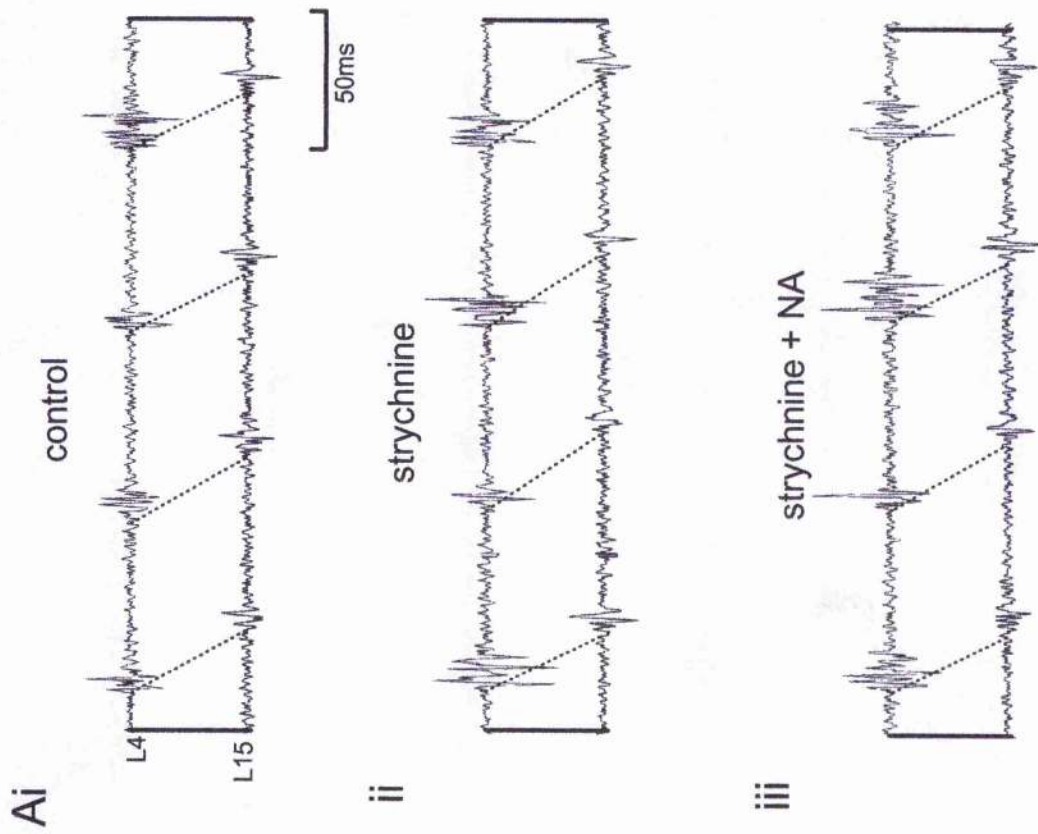


Figure 4.12 The effects of NA on longitudinal co-ordination are substantially occluded by strychnine.

A. Frequency matched excerpts of stage 42 larval fictive swimming activity recorded from L4 and more caudally from L15 in control (i), 10 minutes after the bath application of $1\mu\text{M}$ strychnine and after exposure a 10 minute exposure to NA ($5\mu\text{M}$, iii).

B. Graph plotting longitudinal delay against cycle period for each consecutive cycle of activity in control saline ($n=200$), after exposure to $1\mu\text{M}$ strychnine ($n=114$) and after 10 minutes exposure to $5\mu\text{M}$ NA ($n=46$). In control conditions the correlation between delay and cycle period is highly significant ($p<0.001$) with $r=0.83$. 10 minutes after exposure to $1\mu\text{M}$ strychnine, the correlation between these two parameters of swimming remains significant ($p<0.001$) but is very much weakened ($r=0.57$). The relationship between delay and cycle period is shifted in the presence of strychnine so that delays are longer for any given cycle period. 10 minutes after subsequent addition of $5\mu\text{M}$ NA, a decrease in delay for any given cycle period is incurred, but this decrease is much smaller than when the amine is applied in the absence of strychnine (c.f. figure 3.10). The correlation between delay and cycle period remained significant ($p<0.001$) in the presence of NA with $r=0.63$.

C. Histogram representing the mean delays taken from three episodes of swimming for the same data shown in B. Mean delays significantly ($p<0.001$) increase from 17.4 ± 2.5 ms in control to 21.2 ± 2.2 ms after the bath application of $1\mu\text{M}$ strychnine, whilst delays were decreased significantly ($p<0.001$) to 16.4 ± 2.0 ms upon subsequent addition of $5\mu\text{M}$ NA.



vii) Effect of NA on transected larval swimming in the presence of strychnine.

To establish whether NA remained less effective in the presence of strychnine when inputs from higher centres were removed, experiments were performed on stage 42 *Xenopus* larvae, transected at the level of the first post-otic cleft. Under these circumstances, strychnine (1 μ M) was again capable of occluding the ability of NA (0.5-5 μ M) to increase cycle periods (n=6). Figure 4.13 illustrates an example of the effect of NA on locomotor frequency in the presence of strychnine. Here, cycle periods significantly ($p < 0.001$) decrease from 50.2 ± 3.6 ms in control conditions to 41.7 ± 3.8 ms under 1 μ M strychnine. Subsequent addition of 4 μ M NA resulted in cycle periods significantly ($p < 0.001$) increasing by approximately 15% to an average of 48.2 ± 7.4 ms. It would therefore appear that strychnine can partially, but not completely occlude NA's effects on swim frequency in transected animals.

When NA effects on longitudinal delays were examined in transected animals pre-treated with strychnine, it was found that NA did not significantly decrease delays (n=3). As can be seen in figure 4.14B, the correlation between delay and cycle period is very weak in transected animals. Strychnine (1 μ M) did not appear to have any effect on delays, whilst further addition of NA (5 μ M in this case) also did not have a marked affect on delay magnitude. The lack of effect on delays is shown in the histogram in figure 4.14C which shows that neither strychnine nor subsequent addition of NA significantly ($p > 0.05$) affected delays.

Figure 4.13. Effects of strychnine on cycle periods in the presence of NA in larvae transected at the level of the first post otic cleft.

A. Exerpts of fictive swimming activity recorded from L8 of a stage 42 larvae that had been transected at the level of the 1st post otic cleft in control saline (i), 10 minutes after the bath application of $1\mu\text{M}$ strychnine (ii), and after subsequent 10 minutes exposure to $4\mu\text{M}$ NA (iii).

B. Histogram depicting cycle periods averaged from 20 cycles of activity taken from near the start of 3 separate episodes of swimming under each experimental condition. From these results it can be seen that whilst strychnine significantly decreases cycle periods ($p < 0.001$), further application of NA caused cycle periods to increase (significant, $p < 0.001$), although this increase is smaller than that occurring when NA is applied alone. Mean cycle periods are 50.2 ± 3.6 ms in control saline, 41.7 ± 3.8 ms after exposure to strychnine and 48.2 ± 7.4 ms following further application of NA.

Ai

transected



ii

strychnine



iii

strychnine + NA



B

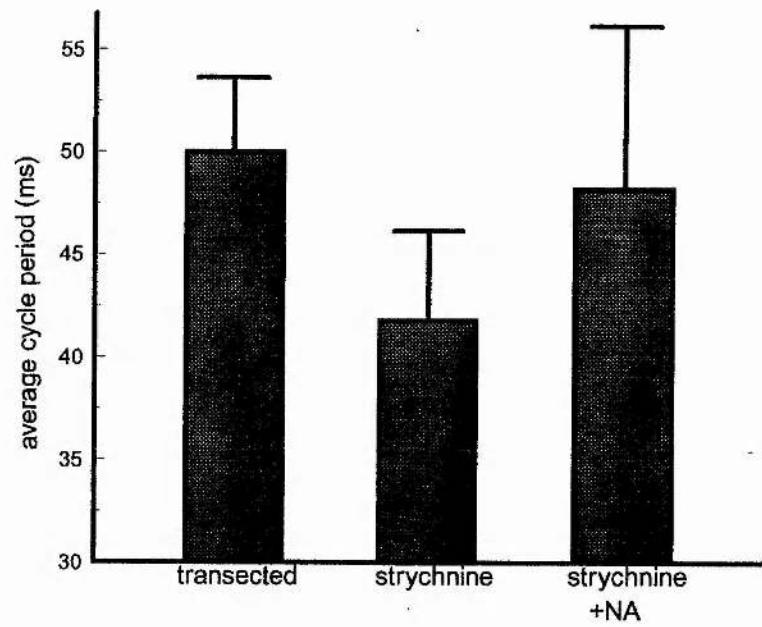
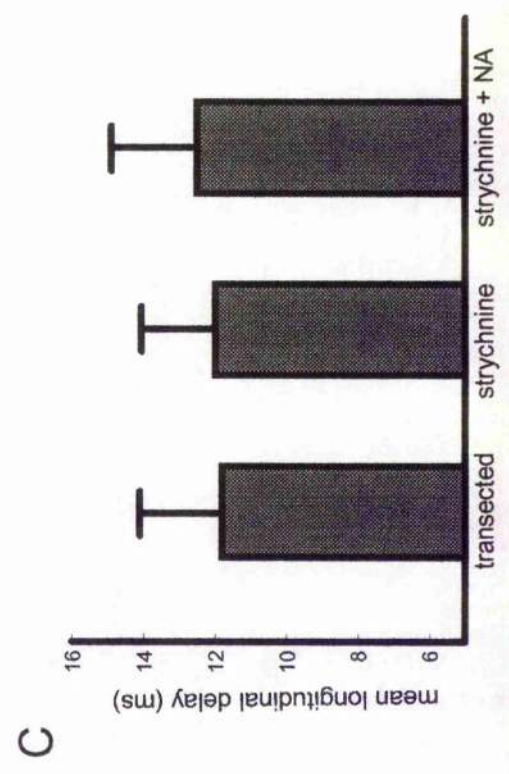
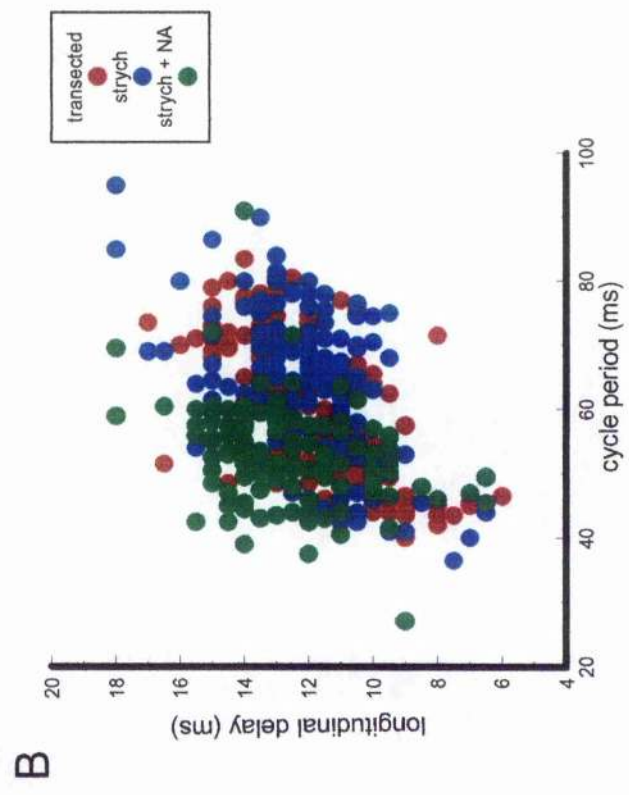
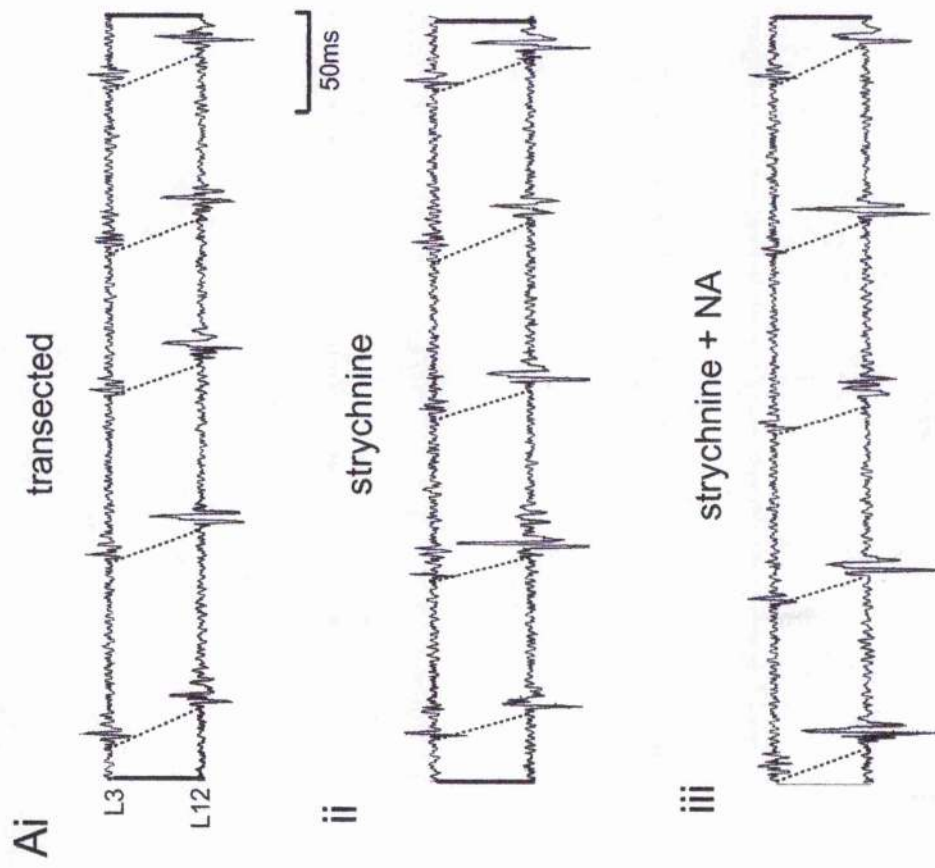


Figure 4.14. Effects of strychnine on longitudinal co-ordination in the presence of NA in larvae transected at the level of the first post otic cleft.

A. Frequency matched excerpts of stage 42 larval fictive swimming recorded from L3 and L12 in control (i), 10 minutes after the bath application of $1\mu\text{M}$ strychnine (ii) and after a 10 minute exposure to $5\mu\text{M}$ NA (iii).

B. Graph plotting longitudinal delay against cycle period for each consecutive cycle of activity within three entire episodes of transected larval swimming in control ($n=126$), after 10 minutes exposure to $1\mu\text{M}$ strychnine ($n=143$) and upon subsequent addition of NA ($n=122$). The correlation between delays and cycle periods is weaker in the transected animal when compared to the intact animal with $r=0.65$. Addition of strychnine weakens the correlation with the r -value now at 0.48 . Further addition of NA does not cause a decrease in delays relative to cycle period, whilst the correlation between delay and cycle period is weakened further ($r=0.30$).

C. Histogram representing the mean delays taken from three episodes of swimming for the same data shown in B where it can be seen that delays do not decrease significantly ($p>0.05$) in the presence of strychnine or after subsequent addition of NA ($p>0.05$). Mean delays are 11.8 ± 2.3 in control, 12.0 ± 2.0 ten minutes after exposure to $1\mu\text{M}$ strychnine and 12.5 ± 2.4 after further addition of $5\mu\text{M}$ NA.



DISCUSSION

i) Can an enhancement of mid-cycle inhibition explain the NA-mediated decrease in swimming frequency?

The modulatory effects of NA on locomotor output have previously been described in only a few vertebrate motor systems, including the adult cat (Barbeau & Rossignol, 1991) and lamprey (McPherson & Kemnitz, 1994, see previous chapter) and little is known of the underlying cellular and synaptic mechanisms through which NA may exert its influence. The data presented in this chapter provide evidence that NA is capable of profoundly affecting glycinergic inhibitory transmission during swimming in *Xenopus* tadpoles. Could the enhancement of glycinergic inhibition explain the ability of NA to modulate locomotor frequency and longitudinal co-ordination? This question will be addressed in this, and the next, section.

With respect to swimming frequency, evidence already exists to suggest that the strength of reciprocal inhibition is important for setting swim speed. The glycine antagonist strychnine has been shown to accelerate the swimming rhythm in *Xenopus* embryos and larvae (Roberts, Dale, Evoy & Soffe, 1985; Dale, 1995b; McDearmid, Scrymgeour-Wedderburn & Sillar, 1997). Similar findings have been reported with low concentrations of strychnine in the lamprey (Cohen & Harris-Warwick, 1984). It would therefore appear that a weakening of reciprocal glycinergic inhibition accelerates the swimming motor pattern. However, the finding that strychnine increases swimming frequency in one half of the sagittally divided *Xenopus* embryo spinal cord indicates that the strychnine-induced increase in swimming speed may be mediated through other mechanisms (Soffe, 1989). Such preparations will lack cross-cord inhibitory connections, and so the

strychnine-induced acceleration in motor output seen in this case cannot be mediated through reciprocal inhibition. One possibility is that a block of ipsilateral inhibition causes an increase in swimming frequency in these preparations. Unfortunately, largely due to a lack of appropriate pharmacological agents, there is presently little experimental information on the effects of strengthening reciprocal inhibitory synapses. Notwithstanding, it has been found that *Xenopus* tadpole swimming frequency is reduced by sarcosine, a glycine reuptake inhibitor (K.T. Sillar & C.A. Reith, personal communication). The effects seen with this agent is remarkably similar to those that I have shown to occur in the presence of NA and adds weight to the hypothesis that the NA-mediated decrease in swimming frequency occurs through a strengthening of mid-cycle glycinergic inhibition.

Aside from the pharmacological findings discussed above, there is supportive evidence from computer simulation studies that the strength of reciprocal inhibition has a dramatic influence on swimming frequency. The most recent and comprehensive computer model of *Xenopus* embryo swimming suggests that changes in inhibitory conductances during swimming are important for determining swim speed (Dale, 1995b) so that small inhibitory conductances produce high frequency swimming whilst larger inhibitory conductances generate low frequency swimming. My results show that NA increases the amplitude and duration of mid-cycle inhibition during swimming and also decreases swimming frequency. I have also shown that a weakening of glycinergic inhibition with strychnine has the opposite effect in that it accelerates swimming frequency. Is it possible that the two phenomena are linked? I found that the duration of the mid-cycle ipsp's during swimming increases as their amplitude increases. On the basis of this observation, it may be that, in the presence of NA, mid-cycle ipsp's become longer in duration and therefore neurones take longer to escape from the inhibition. As such, a delay in the onset of the next cycle of activity would occur.

The converse of this situation would be that a reduction in mid-cycle inhibition allows motoneurons to escape from inhibition sooner and therefore fire earlier, increasing swimming frequency. This would also explain the increased burst durations during larval swimming in the presence of strychnine. From the studies outlined above, there is precedence to suggest that enhancing glycinergic inhibition causes a slowing-down of swimming speed. It is however, worth noting that all intracellular recordings shown in this chapter were made using KCl-filled electrodes. The basis for using this electrolyte was that KCl causes chloride to leak into the cell, therefore reversing the flow of this ion when chloride-gated channels open. This has the result of making glycinergic ipsp's both depolarising and of larger amplitude so that they are easier to study. However, reversing the sign of the mid-cycle inhibition creates an artificial situation within the recorded cell, an effect which could cause a number of potential problems. For example, when using KCl-filled electrodes, the chloride equilibrium potential can change over time due to ongoing chloride leakage into the recorded cell. It is therefore possible that the depolarising mid-cycle ipsp's appear to become larger after exposure to NA simply because of increasing chloride concentrations within the cell rather than a direct effect of the amine. This is, however, unlikely for two reasons. Firstly, cells were given 10 to 20 minutes to stabilise after penetration during which time it was checked that maximal mid-cycle ipsp amplitudes had not changed. Experiments were only performed after this time. Secondly, the effects of the amine on mid-cycle ipsp amplitude were found to be reversible, suggesting that they were due to an effect of the drug rather than an effect of chloride leakage. Another problem when using KCl-filled electrodes arises from the fact that chloride loading artificially adjusts the reversal potential for this ion. Because of this, there are limitations regarding how my results can be interpreted. For NA to exert such strong effects on ipsp amplitude, the sign-reversed mid-cycle inhibition must have often been well below reversal potential during control

conditions in my experiments. However, my results do not allow me to establish if this would also be the case when the inhibition is *hyperpolarising*. It is possible that when the inhibition is hyperpolarising, the inward chloride current is normally much closer to reversal potential on every cycle of swimming during control episodes. If so, then NA would not have such a dramatic effect on the amplitude of hyperpolarising mid-cycle inhibition because it would already be close to maximum amplitude. It is, however, unlikely that this would have been a problem as recordings using KAc-filled electrodes (KAc does not affect the reversal potential of the chloride current) have shown that hyperpolarising mid-cycle ipsp's can vary widely in amplitude during control swimming (K.T. Sillar, personal communication). Nonetheless, it is necessary that recordings using KAc-filled electrodes are now made. It is only when such experiments are performed that it can be established whether NA causes quantitatively similar effects on the amplitude of mid-cycle inhibition when this component of the synaptic drive is hyperpolarising.

If it is to be assumed that NA affects swimming frequency through a strengthening of mid-cycle inhibition then strychnine should block this response. My results have shown that glycine receptor blockade at least partially impairs the ability of NA to reduce swimming frequency. Furthermore, when applied after NA exposure, a substantial reversal in the NA-mediated effects on swimming frequency are seen. However, both the occlusion and reversal of the effects of NA by strychnine were partial in that the amine still had some effect on swimming frequency in the presence of glycine receptor blockade. There are two possible explanations for this. Firstly, the NA-induced slowing of the motor pattern is due only partly to an enhancement of glycinergic transmission, and also relies on other effects. I have obtained evidence to suggest that at least one other transmitter system is affected by NA: under TTX, the frequency of occurrence of GABAergic potentials also appears to increase in the presence of the amine. This is of

particular interest because GABAergic agents have previously been shown to exert a strong influence on the *Xenopus* tadpole swimming frequency so that an increase in GABA receptor activation slows down swimming speed (Reith, 1996). Perhaps the strychnine-resistant effect of NA on cycle period is mediated through an enhancement of GABAergic transmission during swimming. The phasic excitatory drive may also be affected by NA. Modelling studies have shown that by weakening excitatory conductances during swimming, a deceleration in the motor pattern may be achieved (Dale, 1995b). Another possibility is that NA causes de-recruitment of premotor interneurons. The probability interneurons will fire action potentials increases with increasing swimming frequency in the *Xenopus* embryo (Sillar & Roberts, 1993). Following this line of reasoning, it may be that the proportion of interneurons active during swimming, at least in part, helps to determine swimming frequency. Indeed, strychnine which decreases cycle periods has been shown to cause recruitment of premotor interneurons during swimming in the *Xenopus* embryo (Perrins and Soffe, 1996a). Perhaps NA has the opposite effect on interneurone recruitment during swimming. It is quite possible that NA also affects the biophysical properties of neurones within the CPG thereby causing changes in motor output that remain in the presence of inhibitory block by strychnine. For example, modulation of spike capability, resting membrane potential (unlikely as no consistent effects were seen during the course of my experiments), rebound from inhibition or membrane conductance of neurones within the CPG for locomotion could all influence the motor pattern. NA's ability to affect the biophysical properties of cells in the spinal cord have not been studied during the course of my experiments, and the actions of NA at the cellular level now needs to be addressed. Secondly, the other possible reason why NA has residual effects on motor output in the presence of strychnine is that the concentration of strychnine used in my experiments may not have been sufficient to completely block all glycine receptors. Relatively low concentrations of

strychnine were used (1 μ M) during this study in order to minimise non-specific effects which are known to occur at higher concentrations (see introduction). At the dose I used, strychnine may not provide complete block of glycinergic receptors, leaving weakened but not abolished inhibition. The IC₅₀ for the action of strychnine at glycine receptors is 14nM in isolated *Xenopus* embryo motoneurons (Dale, 1995b). Although this value may appear low, it is well to remember that my experiments were performed on whole animals rather than isolated cells. The potency of strychnine in intact preparations appears to be much lower, presumably due to limited access of the drug to the spinal cord. An incomplete block of inhibition could account for the ability of NA to generate some effect on motor output in the presence of strychnine. Higher concentrations could have been used in these experiments to ensure complete block of glycine receptors, but such concentrations may have non-specific effects on various ion channels (see introduction) which are important for shaping motor output, making it difficult to examine the true effects of NA on fictive swimming. It is now necessary to address the first possibility in detail, so the mechanisms through which NA acts can be characterised more completely.

ii) Can an enhancement of mid-cycle inhibition explain the NA-mediated decrease in rostrocaudal delays?

How longitudinal co-ordination is maintained within spinal locomotor networks is still somewhat of an unanswered question. In *Xenopus* embryos, the ability of motoneurons to undergo post inhibitory rebound has been suggested to play a role (Roberts & Tunstall, 1990; Tunstall & Roberts, 1994; Roberts, Tunstall & Wolf, 1995). This hypothesis predicts that mid-cycle hyperpolarisation of motoneurons enables the recovery of motoneurons from their excitation on-

cycle presumably by a removal of sodium channel inactivation and also deactivation of potassium channels. This will have the effect of raising membrane excitability when hyperpolarising input is removed (Tunstall & Roberts, 1994).

Post-inhibitory rebound is a vital component of earlier, first-generation models for embryo swimming (Roberts & Tunstall, 1990; Wall & Dale, 1994; Wolf & Roberts, 1995; Roberts, Tunstall, Wolf, 1995) and it has been suggested to be causal to the generation of the rostrocaudal delays in ventral root activity during locomotion (Tunstall & Roberts, 1994). The degree to which neurones will rebound from inhibition on any given cycle of swimming is presumably proportional to the magnitude of the mid-cycle inhibition. Assuming this premise is correct, neurones which receive stronger mid-cycle inhibition will be more likely to fire due to rebound and this may in turn advance the firing of these neurones relative to those that receive weaker inhibition. There is evidence to suggest that strength of mid-cycle inhibition declines in a rostrocaudal direction along the spinal cord (Tunstall & Sillar, 1993; Tunstall & Roberts, 1994). Indeed Tunstall and Roberts reported that *Xenopus* embryo motoneurones caudal to the twelfth post-otic cleft do not receive any synaptic inhibition at all during swimming. My recordings from embryonic motoneurones in these very caudal regions have suggested that this is not the case in that they all receive glycinergic inhibition on at least some cycles of swimming activity (an example is shown in figure 4.2). There is currently no explanation for the discrepancies in these findings. Nevertheless, my results do support the argument that a rostrocaudal gradient in the strength of reciprocal inhibition does exist in the spinal cord of these animals. As such, post-inhibitory rebound will be more prominent in rostral regions of the spinal cord where the inhibition is strongest. This will raise the excitability of rostral motoneurones as they recover from inhibition, allowing firing in this area to precede caudal motoneurone firing. The outcome of this

gradient of excitability would be to introduce a longitudinal delay in ventral root discharge.

Can the post inhibitory rebound hypothesis help to explain NA's ability to decrease longitudinal delays? I have shown that the amine increases the amplitude of mid-cycle inhibition. In the embryo at least, this effect is much more prominent in caudal regions of the spinal cord where the inhibition tends to be weaker. Following this line of reasoning, global exposure to NA should preferentially enhance the post-inhibitory rebound properties of more caudal motoneurons, allowing them to rebound faster and therefore advance the timing of the excitatory phase relative to rostral regions of the cord. The resultant effect on motor output would be a reduction in rostrocaudal delays, an effect that is seen in the presence of NA. In the larvae, caudal motoneurone recordings have yet to be made, so it is not yet known whether a similar gradient in synaptic inhibition is present in these older animals, although this may well be the case. Nevertheless, an adjustment in the strength of inhibition (and therefore the degree of postinhibitory rebound) is one way in which longitudinal co-ordination could be altered.

There is some experimental evidence that post inhibitory rebound does occur in *Xenopus* embryo motoneurons: when these neurones are held tonically depolarised, they will fire an action potential following brief hyperpolarising current pulses (Roberts, Soffe & Dale, 1986; Soffe, 1990). However, the role of post-inhibitory rebound in rhythm generation is somewhat controversial. First generation mathematical models of *Xenopus* embryo swimming which used biophysical parameters obtained from classical Hodgkin-Huxley equations suggested that rebound from inhibition was crucial for rhythm generation (Roberts & Tunstall, 1990; Wall & Dale, 1994; Wolf & Roberts, 1995; Roberts, Tunstall, Wolf, 1995). In a more recent model for embryo locomotion devised by Dale in 1995, rhythm generation does not depend upon postinhibitory rebound.

This latter model is more comprehensive than its predecessors because it incorporates values for biophysical membrane properties obtained directly from experiments on dissociated *Xenopus* embryo motoneurons. Experimentally, the importance of post inhibitory rebound during swimming is also an unresolved issue. It is unlikely that rebound is essential for rhythm generation because rhythmic motor output still occurs in one half of the sagittally divided spinal cord where cross-cord inhibitory connections that would normally produce rebound will be absent (Soffe, 1989). There is, however, evidence to suggest that rebound can affect motor output during swimming: Tunstall and Roberts found that longitudinal delays increased when strychnine was perfused onto caudal regions of the spinal cord (Tunstall & Roberts, 1991). Furthermore, Green and Soffe (1998) have recently shown that rostral hemisection of one side of the spinal cord (to weaken crossed inhibition in the intact side) also increases the duration of rostrocaudal delays. However, Perrins and Soffe (1996a) found that more local applications of strychnine to caudal cells in the spinal cord advance the timing of motoneurone firing. This report directly conflicts with the post-inhibitory rebound hypothesis, which states that weaker inhibition should delay the onset of motoneurone firing within each cycle of activity. The reasons for these discrepancies have yet to be resolved.

The importance of post-inhibitory rebound remains controversial. Nonetheless, there are other mechanisms through which NA could be acting to exert its effects on longitudinal output. For instance, the amine may directly affect properties of the membrane such as spike threshold. An enhancement in spike capability of caudal neurones would increase the probability of motoneurone firing in this area. NA could also be preferentially enhancing caudal relative to rostral phasic excitation in a similar way to the enhancement of inhibition reported here. Effects on cell properties and the strength of excitatory synapses under NA are areas which now require detailed investigation. What is apparent

from my results is that NA increases the amplitude of mid-cycle inhibition and also decreases longitudinal delays, whilst strychnine both increases delays when applied alone and also reduces the ability of NA to modulate longitudinal co-ordination. Whether the increase in inhibitory synaptic strength seen with NA is responsible for affecting longitudinal co-ordination awaits resolution, but my results suggest that this may be, at least partially, the case.

iii) A possible site for the action of NA.

The fact that NA increases the frequency of TTX-resistant glycinergic ipsp's suggests that the amine acts presynaptically to somehow enhance transmitter release from the terminals of commissural interneurons. Furthermore, this effect of the amine has been shown to persist in the presence of Cd^{2+} (McDermid, Sillar & Scrymgeour-Wedderburn, 1997) which suggests that it occurs independently of any effects on presynaptic Ca^{2+} influx and therefore via a direct effect on the vesicular release machinery. Several laboratories have reported that amines can presynaptically modulate synaptic transmission (e.g. Mintz, Gotow, Triller & Korn, 1989; Wall & Dale, 1993; Shupliakov, Pierbone, Gad & Brodin, 1995). NA in particular has been shown to enhance excitatory synaptic transmission in chick ciliary ganglia, an effect that is thought to be mediated through an increase in the Ca^{2+} sensitivity of the exocytotic process (Yawo, 1996). Furthermore, Kondo and Marty (1997) reported that, in TTX-treated cerebellar stellate cells, NA increases the frequency of occurrence of spontaneous miniature (m) ipsp's without affecting the mean amplitude of these events, an effect which is remarkably similar to that reported here in *Xenopus* motoneurons. Their finding suggested that this process may be mediated through an increase in intracellular cAMP, as cAMP-dependant protein kinase inhibitors blocked the response and

membrane permeant analogues of cAMP and the adenylyl cyclase activator forskolin mimicked it. It may be that the effects of NA on glycinergic transmission in *Xenopus* involve similar mechanisms to those described above and since all identified noradrenergic receptors to date have been shown to be metabotropic, it is reasonable to assume that a G-protein coupled system is involved. Although I did not investigate the involvement of second messenger systems in mediating the NA response, this is an important area for future study. Of the four families of NA receptor, the α_1 -type is positively linked to the phosphatidyl-inositol system, α_2 is negatively linked to cAMP and the β_1 and β_2 positively linked to cAMP. There is now a wide range of pharmacological agents that allow manipulation of these receptors and the second messenger systems to which they couple, which should allow the mechanisms through which NA is acting to be clarified.

iv) Comparisons between the effects of NA and 5-HT on mid-cycle inhibition.

NA is an amine which exerts modulatory effects on a wide range of rhythm generating networks (see chapter 3). Neuromodulators exert their effects by modifying the properties of neurones and their synaptic interconnections, thereby imparting flexibility onto neural circuits that generate behaviour. I have shown here that NA strengthens inhibitory synapses within the spinal cord, an effect which may account for the amines modulatory effects on the swimming motor pattern. This raises the question of whether the CPG also has a means of weakening inhibitory synaptic strengths, so that the amount of inhibition can be adjusted over a wide range to generate a spectrum of output patterns that differ in frequency and intensity. The monoamine 5-HT appears to fit this role. Bath application of 5-HT weakens glycinergic inhibitory synaptic strengths in the

Xenopus tadpole spinal cord, an effect which may occur through a decrease in the probability of the release of glycine from the terminals of commissural interneurons (McDermid, Scrymgeour-Wedderburn & Sillar, 1997). It is conceivable that these two amines may work in concert to finely tune the strength of inhibitory synapses, with release of NA enhancing mid-cycle inhibition and release of 5-HT suppressing it. The modification of swimming behaviour by NA has been described in chapter 3. Its primary effects seem to be to decrease swimming frequency and reduce longitudinal delays whilst having little effect on burst durations. The way in which an up-regulation of mid-cycle inhibition might induce these effects has been discussed above. As 5-HT weakens mid-cycle inhibition, it should have a very different effect on the final output of the motor pattern. When bath applied, 5-HT has relatively little effect on swimming frequency, increases ventral root burst durations and strengthens the correlation between longitudinal delays and cycle period (Sillar, Wedderburn & Simmers, 1992; Tunstall & Sillar, 1993). The response to application of 5-HT is therefore very different from that to NA. Can these changes in the motor pattern be explained by a weakening of mid-cycle inhibition? It has been proposed that by reducing the inhibition, neurones are able to fire more during the excitatory phase of the swimming cycle as the ability of the inhibition to terminate the excitatory phase will be impaired (McDermid, Scrymgeour-Wedderburn & Sillar, 1997). The effect on delays could also be a result of preferentially weakening inhibition rostrally, thereby advancing the timing of motoneurone firing in this region of the spinal cord. Although such contrasting effects could be generated through alterations in the strength of inhibition, it is likely that both amines also affect other properties of the motor network. Indeed, 5-HT has been shown to cause membrane potential hyperpolarisations and voltage-dependant oscillations in *Xenopus* tadpoles (Scrymgeour-Wedderburn, Reith & Sillar, 1997). Other possible effects of NA remain to be investigated. Nonetheless, modulation of

inhibition by the release of the two amines within the spinal cord may help to generate a very flexible and dynamic network for motor control.

v) Functional implications of the effects of NA on GABAergic transmission.

In this chapter, I have also provided evidence that NA enhances the release of GABA presynaptically. It is conceivable that some of the effects on motor output induced by NA are partly due to this effect. There are eight populations of GABAergic neurone in the CNS of the embryo. Two of these populations, the Kölmer Agdhur cells and the ascending interneurons are spinal in origin (Dale, Roberts, Ottersen, & Storm-Mathisen, 1987; Roberts, Dale, Ottersen, & Storm-Mathisen, 1987). The function of both these classes of neurone is unknown. The remaining six GABAergic populations are located in the brain (Roberts, Dale, Ottersen, & Storm-Mathisen, 1987). The function of only one of these populations is known. The mid-hindbrain reticulospinal (mhr) neurones, which originate in the brainstem and descend into the spinal cord appear to halt swimming when activated (Boothby & Roberts, 1992). Although there is little information on the physiological function of the other populations of GABAergic neurone, the effects of pharmacological agents for GABA receptors have been studied in some detail. The neurosteroid 5β -pregnan-3 α -ol-20-one ($5\beta 3\alpha$) which enhances both the presynaptic release and postsynaptic effects of GABA causes a decrease in both the frequency and duration of fictive swimming episodes in both embryos and larvae, although these effects are more pronounced in the larvae (Reith, 1996). Blockade of GABA_A receptors with bicuculline reverses the effects on swimming frequency, but not episode duration (as bicuculline decreases episode length anyway, see Reith, 1996). It may be that at least part of the effects of NA on motor output are attributable to an enhancement of spinal GABAergic

transmission. It is also interesting that NA has opposite effects on the duration of episodes at the two different stages examined in this study. In the stage 37/8 embryo, NA markedly increases episode length whilst in the stage 42 larvae it decreases episode length (see chapter 3). In the stage 42 larvae, this reduction in episode duration was not reversed by blockade of glycine receptors with strychnine. It is therefore unlikely that NA reduces episode length in the larvae by enhancing reciprocal inhibition. Could GABA be involved? Endogenous release of GABA onto spinal motoneurons during locomotion appears to be important for the intrinsic termination of swimming episodes in the stage 42 larvae. In the embryo, GABA does not appear to exert this effect (Reith, 1996). The differences between the two stages may reflect either a less extensive GABAergic innervation of spinal cord at stage 37/8, or a different functional role of GABA between the two stages in development. If GABA release terminates swimming, then an increase in the probability of GABA release by NA could shorten the duration of swimming episodes in the larvae. To clarify this issue, further studies now need to be performed to ascertain the effects of NA in the presence of the GABA_A antagonist bicuculline at both stages of development.

vi) Final comments.

In summary, this chapter has shown that NA increases the amplitude of mid-cycle inhibition during swimming in both the embryo and the larvae of frog *Xenopus laevis*. This effect may occur through a facilitation of glycine release from the presynaptic terminals of commissural interneurons as recordings from motoneurons isolated in TTX have shown that the NA increases the occurrence of spontaneous inhibitory potentials. The effects of NA on reciprocal inhibition may account for a large part of the amines ability to both reduce swimming

frequency and decrease rostrocaudal delays as strychnine can impair the ability of NA to affect the motor pattern. However, it is clear that other mechanisms are also likely to be involved. Nonetheless, alterations in the strength of reciprocal glycinergic inhibition may be a means through which neuromodulators such as NA can affect motor output.

CHAPTER 5

**Initiation of a non-rhythmic motor pattern in *Rana temporaria*
embryos by noradrenaline and nitric oxide**

INTRODUCTION

i) Background.

Aside from shaping the final output of ongoing motor programs (a topic I have addressed in previous chapters), another potential role for transmitters such as NA is in the activation of the spinal networks that generate motor activity. Animals must be able to initiate and terminate particular motor behaviours according to their specific behavioural and developmental requirements. Neuronal inputs originating in the brainstem and descending to the spinal cord may be important for the priming and activation circuits that generate motor activity.

There are three main classes of transmitter which are known to exert a descending excitatory influence on spinal motor networks: these are the excitatory amino acids (EAAs; acting at NMDA, kainate and AMPA receptors), acetylcholine (ACh) and the amines (5-HT, NA and dopamine (DA)). Of these, possibly the most extensively studied are the EAAs. Many brainstem neurones have glutamatergic fibres that descend into the spinal cord (most notably the reticulospinal neurones) and it may be that these are involved in triggering motor activity. However, it should be noted that establishing the function of such neurones is difficult because spinal rhythm-generating networks use glutamate themselves. Therefore, the action of agents that mimic or block the effects of glutamate may occur at the level of the spinal rhythm-generating network itself rather than on descending EAA pathways. Nonetheless, in the lamprey, for example, it has been shown that glutamate can elicit a stable motor pattern for swimming (Cohen & Wallén, 1980; Poon, 1980). This effect is thought to arise primarily from the activation of NMDA and kainate receptor subtypes on spinal neurones (Grillner, McClellan, Sigvardt, Wallén & Wilen, 1981; Brodin, Grillner

& Rovainen, 1985), although AMPA receptors may also play a role (Alford & Grillner, 1990). Similar findings have been made in the *Xenopus* embryo preparation where NMDA, AMPA and kainate receptor activation has also been shown to initiate rhythmic swimming activity (Dale & Roberts, 1984; 1985; Soffe, 1996). With respect to higher vertebrates, locomotor activity recorded in the neonatal rat spinal cord preparation can also be induced by bath application of EAAs and EAA agonists (Smith & Feldman, 1987; Kudo & Yamada, 1987). Homocysteic and cysteic acids produce similar effects (Sqalli-Houssaini, Cazalets, Martini & Clarac, 1993). As homocysteate is found in spinal glial cells, this implicates possible extraneuronal mechanisms in the regulation of motor activity. In the decerebrate cat, EAA agonists can initiate locomotor output and co-administration of NMA with the glutamate uptake blocker dihydrokainic acid (DHK) induces activity that is much more stable than when EAA agonists are applied alone (Douglas, Noga, Dai & Jordan, 1993). Furthermore, locomotion induced by stimulation of the mesencephalic locomotor region (MLR) can be blocked by intrathecal injection of EAA antagonists (Douglas, Noga, Dai & Jordan, 1993). Selective administration of kainate or AMPA receptor agonists does not generate motor responses in this preparation (Douglas, Noga, Dai & Jordan, 1993). EAA's have also been shown to play a role in locomotor initiation in other vertebrate preparations such as the chick (Barry & O'Donnovan, 1987), the rabbit (Fenau, Corio, Palisses & Viala, 1991), the mouse (Hernandez, Elbert & Droge, 1991), the mud puppy (Wheatley & Stein, 1992) and monkey (Hultborn, Petersen, Brownstone & Neilsen, 1993). Kainate and AMPA agonists have been less successful in eliciting stable motor programs in these animals.

The findings summarised above make it tempting to suggest that descending EAA pathways are involved in triggering motor responses. However, it is very likely that EAA agonists used in these experiments directly depolarise neurones within the motor networks to initiate motor activity. At this stage, from

the evidence currently available, it cannot be determined whether exogenous application of EAA agonists elicits motor output by mimicking the action of descending reticulospinal pathways, by globally exciting CPG neurones, or by having an action at both of these levels.

ACh is another transmitter which has been linked to the initiation of motor behaviour. Although there is limited knowledge of the function of ACh in this area, it has been shown to initiate locomotion in the neonatal rat (Smith, Feldman & Schmidt, 1988; Cowley & Schmidt, 1994; 1995) and the *Xenopus* embryo (Panchin, Perrins & Roberts, 1991). However, ACh-induced motor activity is blocked by NMDA receptor antagonists but not nicotinic or muscarinic receptor antagonists in the neonatal rat (Jordan, Brownstone & Noga, 1992). The inference here is that ACh may be important for the initiation of motor responses but is not essential for their maintenance.

The spinal cord of mammals receives innervation from descending aminergic projections which have also been linked to the activation of spinal motor circuitry. The primary dopaminergic pathway to the spinal cord arises in the hypothalamus and descends to the lumbosacral spinal cord (Björklund & Skagerberg, 1982). Information on the role of DA is currently sparse, but it has recently been shown to initiate motor output in the neonatal rat (Smith, Feldman and Schmidt, 1988; Kiehn & Kjaerulff, 1996). More is known of the action of 5-HT. Separate serotonergic pathways descend from the raphe nucleus to the dorsal and ventral horns of the spinal cord in mammals (Björklund & Skagerberg, 1982). Furthermore, some vertebrates, such as humans, monkeys and lampreys, appear to have an extra serotonergic pathway intrinsic to the spinal cord located ventral to the central canal (Van Dongen, Hökfelt, Grillner, Verhofstad & Steinbusch, 1985). Rhythmic alternation between flexor and extensor ventral roots can be evoked by 5-HT in the neonatal rat preparation (Cazalets, Grillner, Menard, Cremieux & Clarac, 1990; Cazalets, Sqalli-Houssaini & Clarac, 1992; Kiehn &

Kjaerulff, 1996) and in the rabbit (Viala & Buser, 1969). However, this effect of 5-HT is not applicable to all vertebrates studied. For instance, the amine does not initiate motor output in *Xenopus* tadpoles (Sillar, Wedderburn & Simmers, 1992) or spinal cats (Barbeau & Rossignol, 1991). In the cat, descending noradrenergic input from the locus coeruleus initiates motor activity. It has been known for over thirty years that the catecholamine precursor L-DOPA can be used to initiate walking in the spinal cat preparation (Janowska, Jukes, Lund & Lundberg, 1967a; Janowska, Jukes, Lund & Lundberg, 1967b; Fossberg & Grillner, 1973). More recent studies have suggested the effects of L-DOPA are mediated through α_2 noradrenergic receptors since intrathecal injection of NA (Kiehn, Hultborn & Conway, 1992) and intraperitoneal injection of the α_2 agonist clonidine (Barbeau & Rossignol, 1991) produce effects analogous to those seen with L-DOPA. However, pharmacological depletion of NA does not prevent the initiation of locomotion in the cat (Steeves, Schmidt, Skovgaard & Jordan, 1980) indicating that this amine is not essential for generating motor output. Nonetheless, clonidine has been shown to accelerate locomotor recovery in spinal cats (Barbeau, Chau & Rossignol, 1993) and such findings have highlighted potential clinical applications of noradrenergic drugs in the treatment of spinal injuries. Indeed, clinical trials have reported that clonidine also improves motor capability in patients with paretic spasticity (Stewart, Barbeau & Gauthier, 1991).

The information discussed above suggests that descending spinal inputs from the brainstem may be involved in the initiation of motor programs and in some vertebrates at least, these effects may be mediated through the release of amines such as NA.

ii) The function of nitric oxide as a transmitter and its possible link to NA action.

NO is a free radical gas that has only recently been identified as a putative neuronal messenger. Unlike conventional transmitters, NO is not stored in vesicles but secreted both pre and postsynaptically from nerves through a Ca^{2+} -dependent mechanism. Due to its gaseous nature, NO is highly membrane permeable and, rather than acting through the classical receptor-mediated signal transduction pathways, NO can diffuse directly into cells, causing conformational and functional changes in heme-containing target proteins such as guanylyl cyclase.

The discovery that NO acts as an intercellular signalling molecule came about in the late 1980's. It has long been known that ACh and other transmitters can induce smooth muscle relaxation. The observation that rabbit aorta relaxations induced by ACh were dependent on the presence of endothelial cells (Furchgott & Zawadski, 1980) led to the discovery that they release a diffusable messenger capable of maintaining vascular tone. This messenger was termed endothelial-derived relaxing factor (EDRF). Later it was found that NO donors (such as glyceryl trinitrate and sodium nitroprusside) could elicit relaxation in a manner similar to EDRF when endothelial layers were removed from smooth muscle. The actions of both EDRF and NO could also be blocked by haemoglobin and generators of superoxide ions. These findings, coupled with the discovery that EDRF and NO had very similar half-lives (4-6s), led to the suggestion that EDRF was in fact NO. This hypothesis was confirmed in 1987 when it was found that vascular endothelial cells release NO in quantities that could account for the actions of EDRF (Ignarro, Buga, Wood, Byrns & Chaudhuri, 1987; Palmer, Ferrige & Moncada, 1987). From this pioneering work NO has been established as a regulator of many biological processes and functions such as intestinal

relaxation (Desai, Sessa & Vane, 1991), penile erection (Rajfer, Aronson, Bush, Dorey & Ignarro, 1992), neurotoxicity (Dawson, Dawson, London, Bredt & Snyder, 1991), hippocampal and cerebellar plasticity (Odell, Hawkins, Kandel & Arancio, 1991; Bohme, Bon, Stutzmann, Doble & Blanchard, 1991; Schuman & Madison, 1991; Haley, Wilcox & Chapman, 1992), vision (Fesenko, Kolesnikov & Lyubarsky, 1985) and circadian rhythmicity (Ding, Chen, Weber, Fairman, Rea & Gillete, 1994).

NO is synthesised by the enzyme nitric oxide synthase (NOS), of which several subtypes have now been identified. All forms of NOS catalyse the oxidisation of L-arginine, giving rise to stoichiometric amounts of L-citrulline and NO. Several heme-containing proteins are modified by NO which causes a functional change in these molecules. The most widely studied of these is guanylyl cyclase which, when activated by NO, catalyses the conversion of GTP to cGMP. The role of cGMP has not been fully established in most systems but it is known to affect several biological processes such as smooth muscle relaxation, inhibition of platelet aggregation, and retinal signal transduction. cGMP regulates a variety of protein kinases and phosphodiesterases (see Lincoln & Cornwell, 1993), such as the cGMP kinases whose substrates include cytoskeletal proteins (Baltensperger, Chiesi & Carafoli, 1990), G_o proteins which are involved in activation of Ca^{2+} -ATPase pumps (Yoshida, Sun, Cai & Imai, 1991), G_i proteins associated with the cytoskeleton (Baltensperger, Chiesi & Carafoli, 1990) and sarcoplasmic reticulum Ca^{2+} -ATPase regulatory proteins (Raeymaekers, Hofmann & Casteels, 1988). There is also a growing body of evidence that suggests cGMP has a direct action on ion-channel properties. For example, in the retina, cGMP directly activates a sodium channel (Fesenko, Kolenikov & Lyubarsky, 1985), and it now appears that cGMP-regulated channels exist in a variety of other tissues (Biel, Altenhofer, Hullin, Ludwig, Freichel, Flockerzi, Dascal, Kaupp & Hofmann, 1993).

There has only been one report to date of NO affecting motor activity. In 1997, Keef and colleagues demonstrated that NO activates an oscillatory motor pattern in the canine colon (Keef, Murray, Sanders & Smith, 1997). Nonetheless, it is now clear that NO is a ubiquitous messenger in both peripheral and central nervous systems. In vertebrates, NOS is located in neurones in the spinal cord of many species including the cat, rat, mouse, primate and amphibian specifically in the dorsal horn, although ventral horn motoneurons have also been shown to contain NOS in the human spinal cord (Springall, Springall, Riverosmoreno, Buttery, Suburo, Bishop, Merrett, Moncada, & Polak, 1992; Terenghi, Riverosmoreno, Hudson, Ibrahim, & Polak, 1993; Br uning & Mayer, 1996).

NO has also been implicated in the regulation of catecholamine release in several systems. The effects appear to be specific for the tissue and animal studied. For example, in bovine chromaffin cells, rat medial basal hypothalamus, rat sympathetic nerves and the guinea pig vascular tree, NO synthesis tonically suppresses the release of catecholamines (Schwarz, Rodriguez-Pascual, Koesling, Torres & Forstermann, 1998; Seilicovich, Lasaga, Befumo, Duvilanski, Diaz, Rettori & McCann, 1995; Schwarz, Diem, Dun & Forstermann, 1995; Addicks, Bloch & Feelisch, 1994). Conversely, in the anorectum of the possum, the rat mesenteric artery and the rat striatum, NO appears to facilitate release of catecholamines (Thatikunta, Chakder & Rattan, 1993; Yamamoto, Wada, Asada, Yanagita, Yui, Niina, Sumiyoshi, Kobayashi, & Lee, 1997; Stewart, Michel, Black & Humphrey, 1996). Such findings coupled with the lack of knowledge of the role of NO in motor control provided the impetus to investigate the action of these transmitters on *Rana* embryo motor behaviour. I have already shown that NA does not initiate locomotion in *Xenopus* tadpoles (see chapter 1) and so I examined the role of NA and NO in eliciting locomotion in another amphibian preparation: the stage 20 embryo of the frog *Rana temporaria*. This animal was chosen for study for three main reasons. Firstly it is capable of generating well co-

ordinated motor output that is amenable to study and has already been well characterised at both the behavioural and electrophysiological level (Soffe, 1991a; Soffe & Sillar, 1991). Secondly, the motor activity of this embryo is much more complex and variable than that of the *Xenopus* embryo, which makes its behaviour closer to that of adult vertebrates (see chapter 1 for description). Finally, both the *Rana* and *Xenopus* embryos are closely related animals that are at a similar stage in development, allowing comparisons between the effects of transmitters such as NA in two similar preparations to be made.

RESULTS

i) Induction of a non-rhythmic motor pattern by NA.

The bath application of 1-10 μ M NA reliably induces a non-rhythmic pattern of motor activity in *Rana* embryos (Figure 5.1). Around 2 minutes after the bath application of NA (10 μ M), a series of spontaneous bursts of ventral root discharge occurred. The motor bursts varied in duration from single spikes to prolonged bursts lasting around 2 seconds and were indistinguishable from those seen to occasionally occur in control conditions in some preparations. As the recordings made from opposite sides of the body in Figure 5.1A shows, motor bursts never occurred synchronously across the two sides of the body. Thus the motor pattern always displayed left-right alternation but was non-rhythmic (n=2). A wash to control saline would also cause cessation of the NA-induced motor pattern (not shown). Furthermore, if NA was left in to recirculate in the saline, the pattern of activity would cease after around two to eight minutes, even though the amine was still present.

When recordings were made from ventral roots at rostral and caudal positions on the same side of the body (n=10), the NA-induced motor activity was found to occur as waves of discharge that propagated in a rostrocaudal direction with a brief delay between myotomal muscle segments. Figure 5.1B shows an excerpt of activity from an experiment where recordings were made from the 3rd and 11th intermyotomal clefts on the left side at around 3 minutes after exposure to 10 μ M NA. The dotted lines represent the brief rostrocaudal delay in the onset of ventral root bursts at the two locations.

In some instances bursts of discharge elicited by NA could occasionally develop into a brief period of rhythmic swimming. Such an effect occurred

Figure 5.1. NA induces a non-rhythmic motor pattern in *Rana* tadpoles.

A. Motor responses recorded two minutes after the bath application of NA ($10\mu\text{M}$) from electrodes placed over the 3rd post otic cleft on the right side (R3) and the 4th post otic cleft on the left side (L4) of the body (i). Note that motor output does not occur in a rhythmic fashion. Aii shows an expanded excerpt of activity taken from boxed area in Ai where it can be seen that motor output alternates between the two sides.

B. Ipsilateral motor responses recorded at around three minutes after the bath application of $10\mu\text{M}$ NA, rostrally from the 3rd (L3) and more caudally from the 11th (L11) post otic clefts on the left side of the body. Note the brief rostrocaudal delay between the onset of bursts in the two ventral roots (delays depicted by dotted lines).

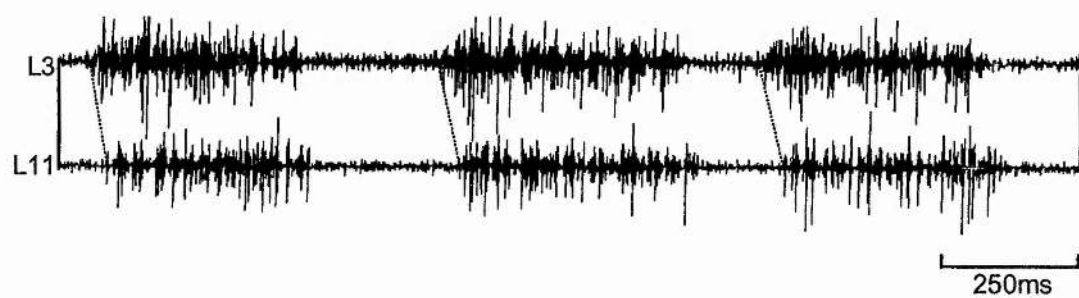
Ai



ii



B



relatively infrequently. An example of this effect is illustrated in figure 5.2. In this preparation, no spontaneous activity was recorded under control conditions (figure 5.2Ai). 3 minutes after bath application of $10\mu\text{M}$ NA, bouts of rhythmic motor behaviour similar to those previously reported as suitable for driving swimming behaviour (Soffe, 1991a) were recorded (figure 5.2Aii, B).

Periods of spontaneous activity elicited by NA commonly lasted around 2-8 minutes, during which time the co-ordination and occurrence of the bursts induced by the amine appeared to be random. This is illustrated in figure 5.3, where the interval between consecutive bursts of ventral root discharge recorded from the left and right sides of the body are plotted against time. Normally a wash back to control saline lasting at least 20 minutes was required before the response to re-application of NA could be reinstated (not illustrated).

In order to investigate synaptic drive to motoneurons during the NA-induced motor pattern, intracellular recordings were made from presumed motoneurons in the ventral quarter of the spinal cord using KCl filled microelectrodes. Under these conditions, the motor programme initiated by NA was found to be characterised by phasic depolarisations which commonly occur in phase with the ventral root discharge and were often sufficiently large to trigger impulses ($n=6$, figure 5.4Aii, B). This suggests that they are likely to arise from some form of excitatory input to motoneurons. In some instances depolarisations would also occur when the ipsilateral ventral roots remained quiescent (E.g. figure 5.4Ai and 5.5Bii). The most likely explanation for this is that these potentials are sign-reversed depolarising ipsp's arising from activity in contralateral commissural-like interneurons on the opposite side of the spinal cord.

Dimming of the illumination can sometimes induce bouts of non-rhythmic ventral root discharge (Soffe & Sillar, 1991; see introduction), similar to those seen under NA. Figure 5.5 shows comparisons of the light dimming response and

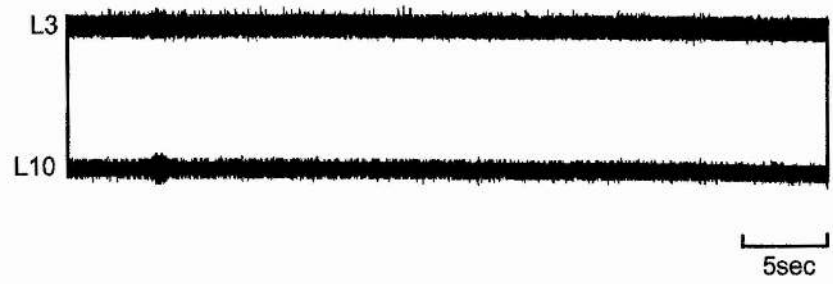
Figure 5.2. Rhythmic motor output induced by NA.

A. Ventral root recordings made from ventral root clefts L3 and L10 in control saline (i) and around 3 minutes after exposure to $10\mu\text{M}$ NA (ii). It can be seen that in this example, NA induces spontaneous rhythmic motor activity similar to that reported as suitable for driving swimming activity in these animals (Soffe, 1991a).

B. Expanded excerpt of activity taken from the boxed area in Aii to show more clearly that the initial prolonged burst of ventral root activity is followed by rhythmic output that occurs down the length of the animal with a brief longitudinal delay between ventral root clefts.

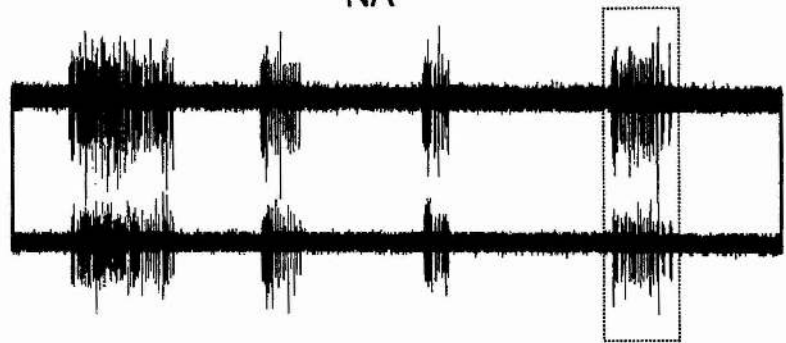
Ai

control



Aii

NA



B



Figure 5.3. Frequency plot of interburst intervals during the NA-induced motor pattern.

The interval between each burst of ventral root activity occurring in clefts L4 and R3 plotted against time for an entire episode of motor activity induced after bath application $10\mu\text{M}$ NA.

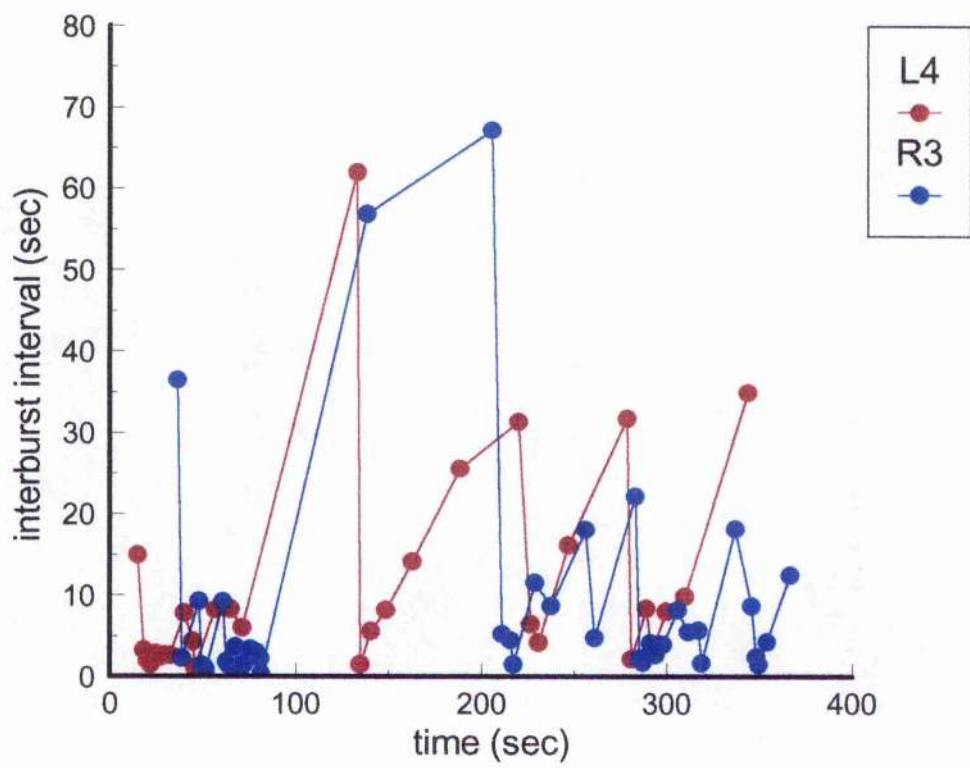


Figure 5.4. Synaptic drive to motoneurones during non-rhythmic motor output induced by NA.

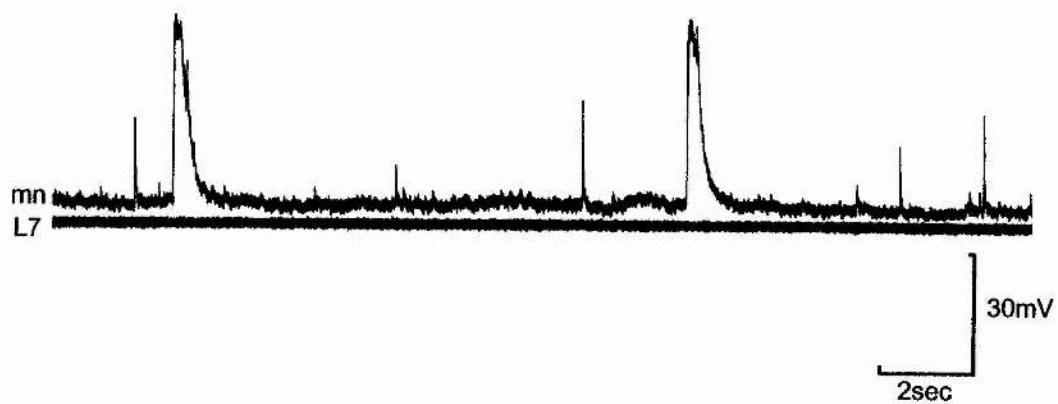
Intracellular recordings made with a KCl-filled electrode from a motoneurone (mn) located at the level of the 3rd post otic cleft in control saline (Ai) and 4 minutes after the bath application of $10\mu\text{M}$ NA (Aii, B).

A. Under control conditions, motoneurones receive little phasic synaptic input and there is no activity in the recorded ventral root cleft (L7) (i). Bath application of $10\mu\text{M}$ NA induces phasic depolarising potentials in motoneurones which are accompanied by bursts of ventral root output (ii).

B. Expanded trace of part of the activity recorded in Aii (dotted lines in Ai represent expanded area).

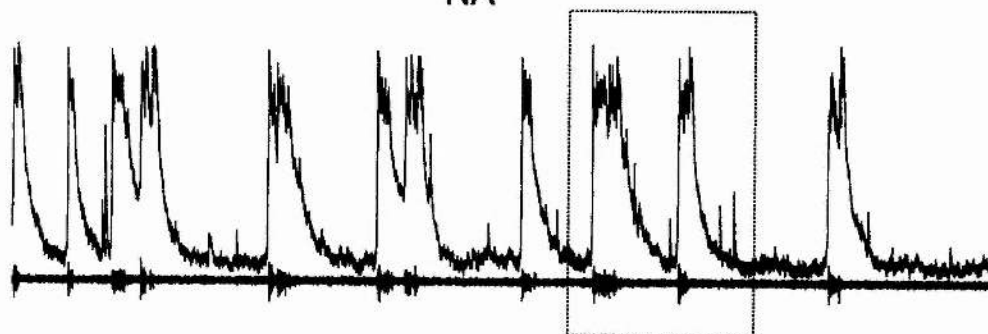
Ai

control

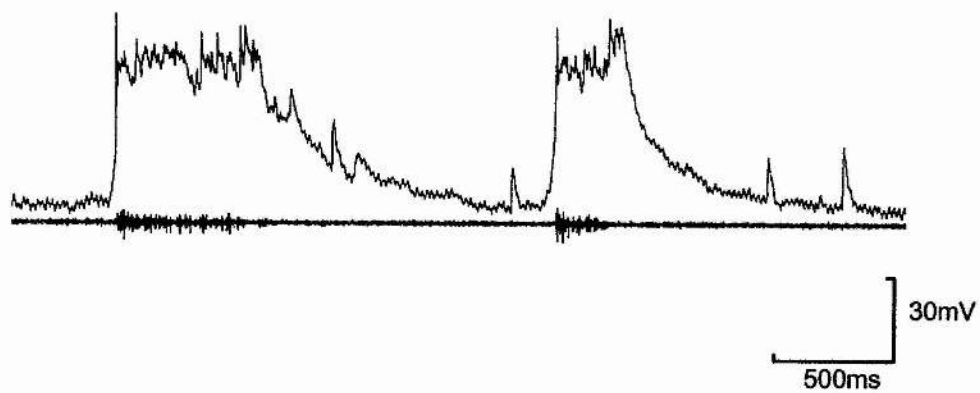


ii

NA



B



the response initiated three minutes after the bath application of 10 μ M NA. The motor pattern elicited in response to dimming of the illumination (Ai) is very similar to that induced by bath application of NA (Aii). Furthermore, when recordings were made using KCl-filled electrodes, it was found that the synaptic drive to motoneurons during activity induced by light dimming (Bi) was also very similar to that induced by NA (Bii). The amine also affected the dimming response in two ways. Firstly, it increased the probability that dimming of illumination would induce a response. Secondly, it increased the duration of the response to light dimming. This effect is illustrated in figure 5.6 where under control conditions a single burst of ventral root discharge occurs after the illumination is dimmed (figure 5.6A). 4 minutes after addition of NA (10 μ M), light dimming induces a more prolonged bout of ventral root activity which occurs with a reduced latency to onset (figure 5.6B).

ii) Pharmacology of the NA response.

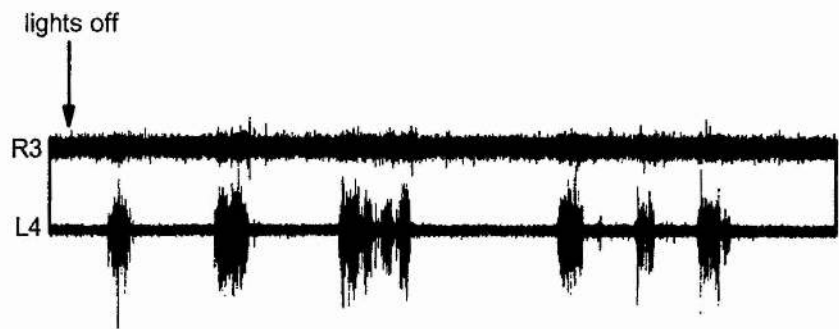
In order to investigate which adrenergic receptors mediate the response to NA, *Rana* embryos were pre-exposed to either the α_1/α_2 antagonist phentolamine, or the β_1/β_2 antagonist alprenolol before addition of NA. These broader spectrum antagonists were chosen for use rather than more specific antagonists because of concern over the differences in pharmacology between amphibian NA receptors and mammalian NA receptors (O'Donnell and Wanstall, 1982). The results were somewhat inconclusive as it was found that phentolamine (20-100 μ M) could block the NA response in only 1 of 6 preparations tested whilst alprenolol (20-200 μ M) was able to block the NA response in the majority of preparations (5 of 9). The pharmacology of the NA response is therefore not fully resolved, although β -receptors seem likely to be involved. An example of the

Figure 5.5. Similarity between the NA-induced non-rhythmic motor pattern and the response to dimming of illumination.

A. Ventral root recordings made from R3 and L4 after dimming of illumination in control saline (i) and after 3 minutes exposure to $10\mu\text{M}$ NA under constant illumination (ii). Note similarity between the ventral root discharge under each condition.

B. Intracellular recording made with a KCl-filled electrode from a motoneurone (mn) located near L5 after dimming of illumination in control saline (i) and after 3 minutes exposure to $10\mu\text{M}$ NA in the presence of constant illumination (ii). Dimming of the illumination in control saline evokes bursts of discharge in the recorded ventral root (L9) that are coincident with depolarisations in the recorded motoneurone. Bath application of $10\mu\text{M}$ NA in the same preparation causes a pattern of synaptic activity that is similar to the dimming response shown in Bi.

Ai

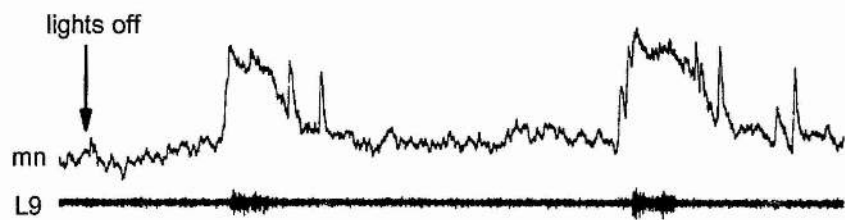


ii

NA



Bi



ii

NA

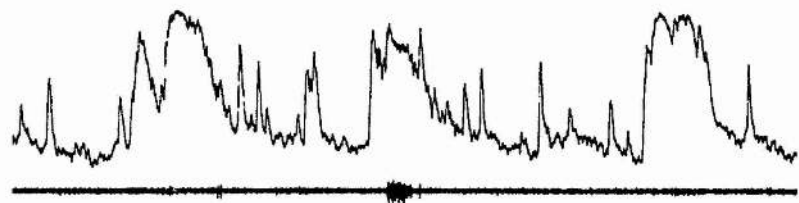
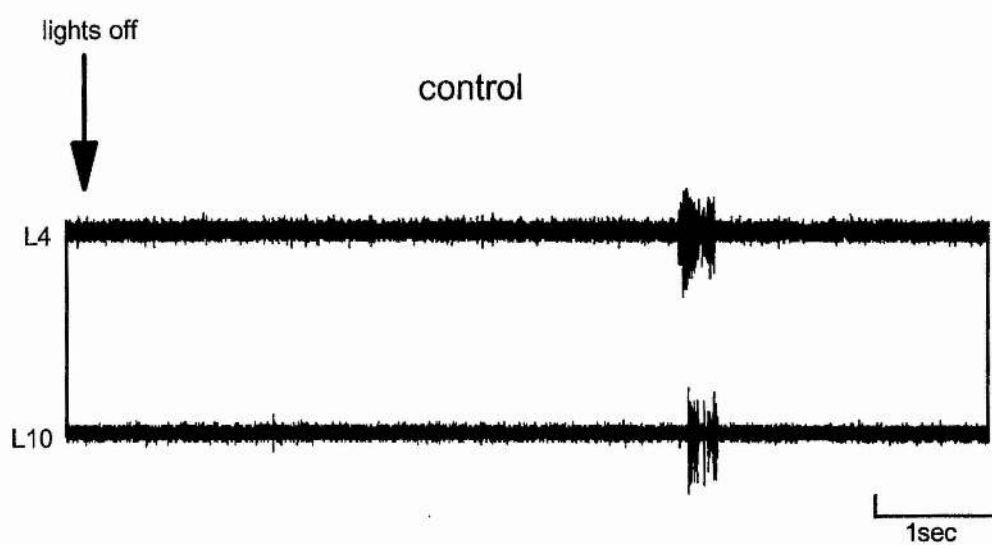


Figure 5.6. NA enhances the response to dimming of illumination.

A. Ventral root activity recorded from L4 and L10 after dimming of illumination in control saline. Light dimming induces a motor response comprising a single burst of ventral root discharge in this case at a latency of around 4.5 seconds.

B. Response to dimming of illumination recorded from the same preparation as shown in A after 4 minutes exposure to 10 μ M NA. Dimming of illumination now generates much more intense and prolonged periods of ventral root activity, occurring at a latency of around 2 seconds.

A



B

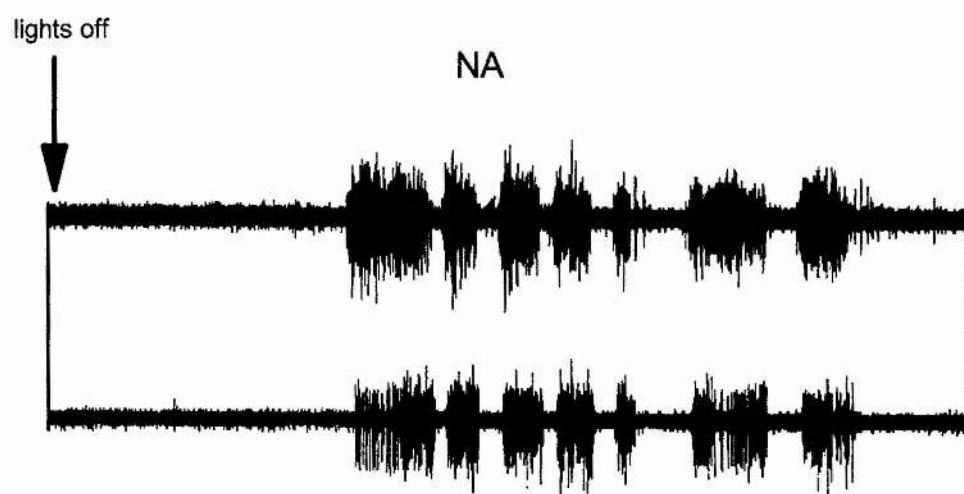
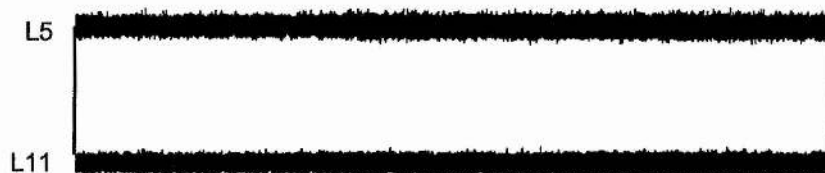


Figure 5.7. The response to NA can be blocked by alprenolol.

A. Ventral root recordings made from L5 and L11 show no motor activity in control saline. B. After twenty minutes exposure to β -antagonist alprenolol ($30\mu\text{M}$), the ventral roots remain silent. C. Subsequent addition of $10\mu\text{M}$ NA in this presence of alprenolol does not induce spontaneous motor output. D. After a wash for 20 minutes in control saline, two minutes after re-application of $10\mu\text{M}$ NA bursts of ventral root discharge are seen to occur.

A

control



B

alprenolol



2sec

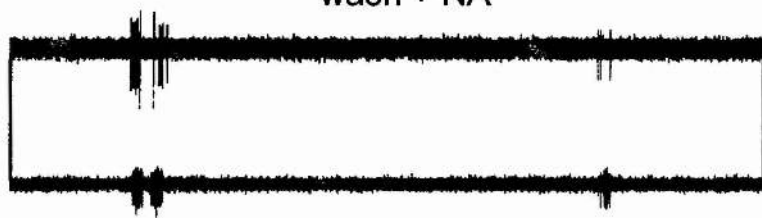
C

alprenolol + NA



D

wash + NA



antagonistic action of alprenolol is shown in figure 5.7. Here it can be seen that under control conditions no spontaneous bursts of discharge occur (A). Similarly, motor output is not initiated by alprenolol (B) or when NA is added in the presence of this antagonist (C). However, re-application of NA after a 20 minute wash to control saline resulted in the generation the non-rhythmic motor response that is produced by the amine (D).

Since the NA response may have involved activation of both α and β adrenoceptor subtypes, a series of experiments were performed where phentolamine (100 μ M) and alprenolol (100 μ M) were co-applied to preparations before subsequent exposure to NA. It was found that under these conditions the co-application of both antagonists did not block the response to NA in any of the preparations tested (n=3, not illustrated). The pharmacology of the NA response therefore remains to be fully elucidated.

iii) Effects of NA on fictive swimming in *Rana* embryos.

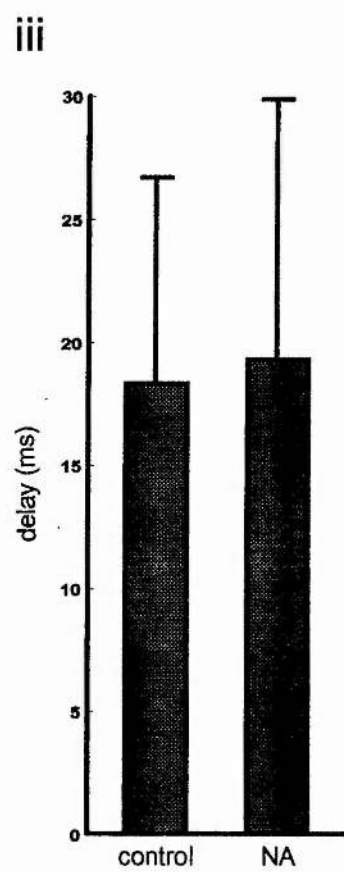
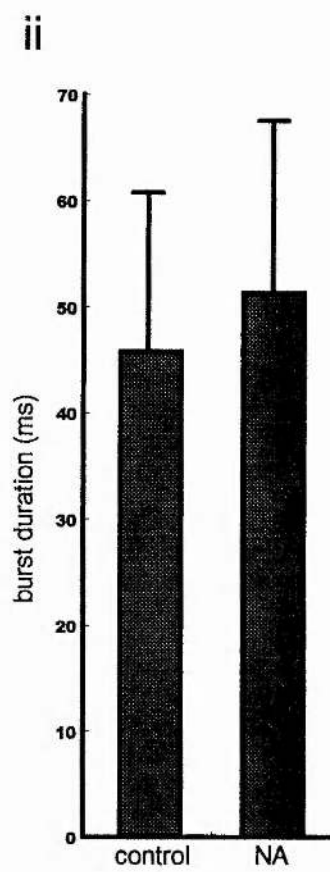
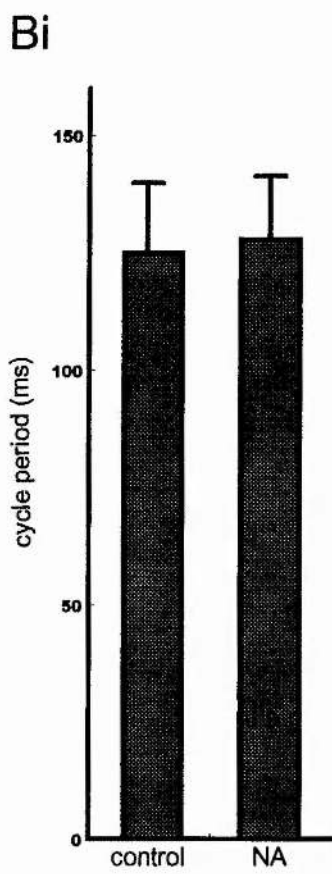
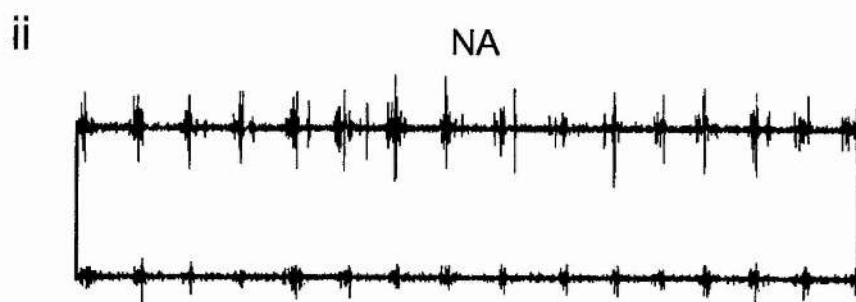
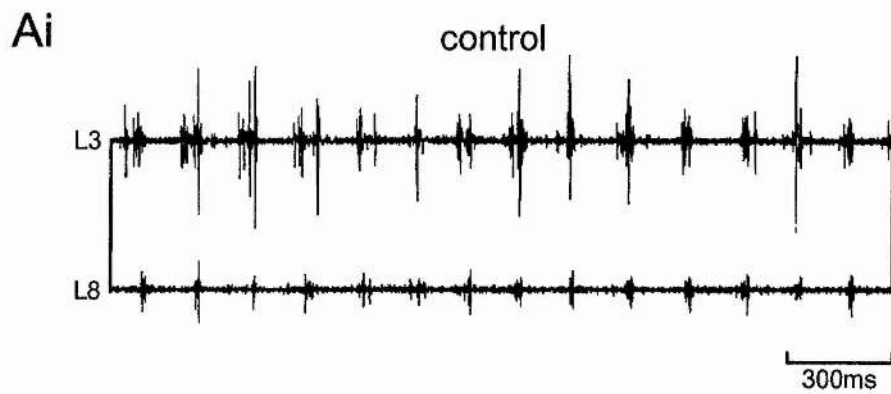
The preceding results illustrate that NA can trigger a non-rhythmic motor pattern that resembles the response to dimming of illumination. NA does not usually trigger fictive swimming but the possibility that the amine might modulate ongoing swimming, as it does in *Xenopus*, was important to determine.

The effects of NA on the three main parameters (burst duration, cycle period and longitudinal delay) of fictive swimming episodes evoked by electrical stimulation were examined in *Rana* embryos (n=5). When data was pooled from three complete episodes under each condition, it was found that none of these parameters were significantly ($p>0.05$) altered after the bath application of NA (1-20 μ M). This lack of effect is shown in the histograms in figure 5.8 which illustrates the average burst duration, cycle period and longitudinal delay taken for

Figure 5.8. NA does not modulate fictive locomotor activity in *Rana*.

A. Exerpts of ventral root activity recorded near the beginning of an episode of fictive locomotion from L3 and L8 in control saline (i) and 7 minutes after bath application of 10 μ M NA (ii). Motor output was elicited by stimulation of the tail skin (see chapter 2). NA does not obviously affect frequency, burst duration or longitudinal co-ordination during fictive swimming.

B. Histograms of data measured from the same preparation shown in A. NA does not significantly ($p > 0.05$) affect cycle period (i), burst duration (ii) or longitudinal delay (iii).



3 whole episodes under each experimental condition. NA (seven minutes exposure at $10\mu\text{M}$ in this instance) had no significant effect on swimming.

iv) The nitric oxide donor SNAP induces a similar non-rhythmic motor pattern to NA.

In order to study the effects of NO on *Rana* embryos, the NO donor S-nitroso-n-acetylpenicillamine (SNAP) was bath applied to immobilised preparations. SNAP ($100\mu\text{M}$) was found to induce a motor pattern that was very similar to that elicited by NA. Figure 5.9 shows a typical response to the NO donor. After around 3 minutes exposure to SNAP ($100\mu\text{M}$), bouts of non-rhythmic motor activity characterised by ventral root activity that varied in duration from single spikes to prolonged bursts lasting around 2 seconds in duration were seen to occur. Such bouts of activity lasted for around 2-8 minutes. The activity in figure 5.9A, recorded simultaneously from ventral roots on the left and right sides of the body, shows that the activity is non-rhythmic and always alternating, rather than synchronous on the two sides of the body ($n=3$). Recordings made from ventral roots at different positions on the same side of the body ($n=9$, figure 5.9B) show that the response occurs as a wave of ventral root discharge that progresses rostrocaudally with a brief delay between ventral root clefts (delays depicted by dotted lines). The effect of SNAP was readily reversed by wash to control saline (not illustrated). In some instances bursts of discharge induced by SNAP were followed by a brief period of rhythmic swimming. These episodes were always preceded by a burst of non-rhythmic motor activity. Furthermore, unlike the non-rhythmic motor response which always occurred upon exposure to SNAP, swimming activity was uncommon. An example of this is shown in figure 5.10 where around 4.5 minutes after bath application of $100\mu\text{M}$

Figure 5.9. SNAP induces a non-rhythmic motor pattern in *Rana* embryos.

A. Motor responses recorded from R8 and L5, three minutes after the bath application of SNAP (100 μ M). Note that motor output is non-rhythmic and never occurs synchronously across the two sides of the body. The lack of synchrony is more clearly illustrated in the expanded excerpt of activity shown in Aii which is taken from the area boxed in Ai.

B. Ipsilateral motor recordings made from rostrally located L3 and more caudally located L8 post otic clefts after a 2.5 minute exposure to 100 μ M SNAP. Note the brief rostrocaudal delay between output of the two ventral roots (delays represented by dotted lines).

Ai



ii



B

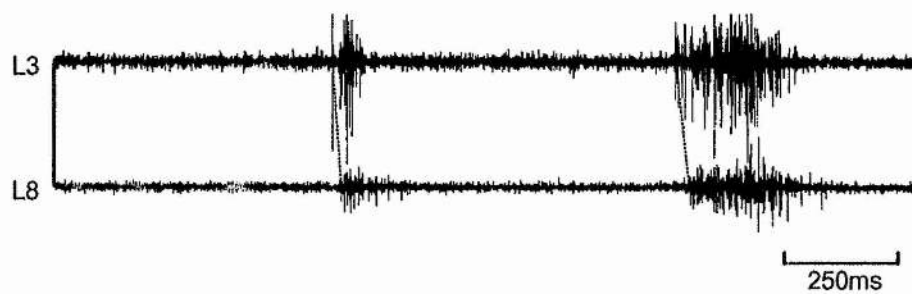


Figure 5.10. Rhythmic motor output induced by SNAP.

- A. Ventral root recordings made from L5 and R8 in control (i) and 4.5 minutes after exposure to 100 μ M SNAP (ii). It can be seen that in this example SNAP could elicit rhythmic motor activity similar to that reported as suitable for driving swimming activity in these animals.
- B. Expanded trace of the activity boxed in Aii showing that motor activity is rhythmic and occurs in an alternating fashion across the two sides of the body.

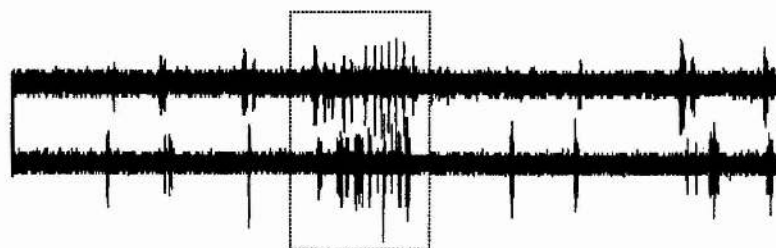
Ai

control



Aii

SNAP



B



SNAP a burst of spontaneous ventral root discharge developed into a bout of rhythmic swimming activity. The SNAP induced spontaneous activity had a very similar time-course to that of NA, lasting around 2-8 minutes, with the co-ordination and occurrence of the bursts appearing to be random (see figure 5.11).

In order to test for possible non-specific actions of SNAP, n-acetylpenacillamine -an inactive analogue of SNAP- was bath applied to *Rana* embryos. This compound was found to be unable to initiate bouts of motor activity at concentrations equivalent to those used for SNAP (100 μ M, n=4, see figure 5.12), even after it had been allowed to recirculate in the ring for over 10 minutes. These results indicate that the ability of SNAP to induce motor output are due to the release of NO rather than a non-specific action of SNAP.

Intracellular recordings from motoneurons using KCl-filled microelectrodes revealed that the bursts of ventral root discharge induced by 100 μ M SNAP application were accompanied by phasic depolarisations in motoneurons (n=9, figure 5.13Aii, B). Again, in some instances motoneurons received depolarising input whilst the ventral roots were silent. Such potentials are most likely to be sign-reversed inhibitory potentials produced by activation of inhibitory interneurons on the opposite side of the cord.

The similarity between the SNAP response and the light dimming response was also examined. Figure 5.14 demonstrates an excerpt of ventral root activity following light dimming (figure 5.14Ai) and around 3.5 minutes after exposure to SNAP (figure 5.14Aii). It can be seen that the response induced by SNAP exhibits a marked resemblance to that produced by dimming of illumination in control saline. It was also found that the synaptic drive seen in motoneurons after dimming of illumination (figure 5.14Bi) was very similar to that induced by SNAP (figure 5.14B). Like NA, SNAP increased the probability that ventral root output would occur upon dimming of illumination as well as the duration and intensity of this response. An example of this effect is illustrated in

Figure 5.11. Frequency plot of interburst interval during the SNAP-induced motor pattern.

The interval between each burst of ventral root activity recorded from L5 and R8 plotted against time for an entire episode of non-rhythmic motor activity induced by 100 μ M SNAP. It can be seen here that the ventral root discharge does not occur rhythmically.

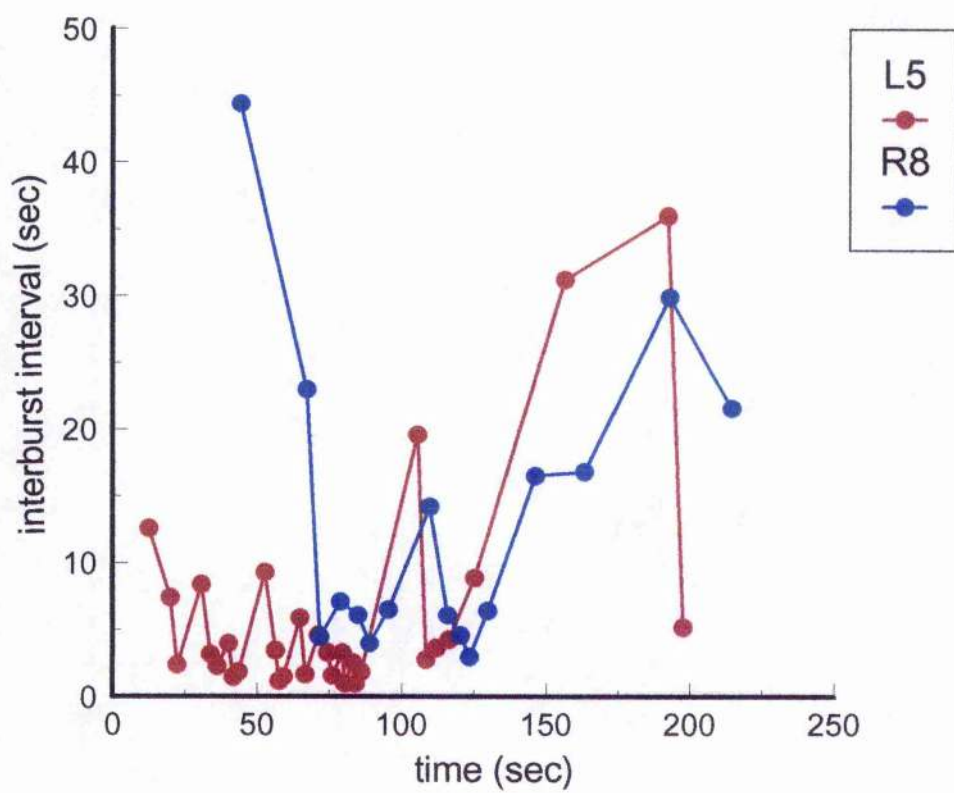


Figure 5.12. N-acetyl-penicillamine does not induce motor activity.

- A. Lack of ventral root activity recorded from post otic cleft L9 shows that in control conditions, no ventral root discharge occurs.
- B. Four minutes after addition of 100 μ M n-acetyl penicillamine, the ventral root remains quiescent.
- C. In the same preparation, after a 20 minute wash in control saline, 3 minutes after exposure to 100 μ M SNAP, a non-rhythmic pattern of motor discharge is induced.

A


control

L9 

B

n-acetyl-penicillamine




3sec

C

SNAP



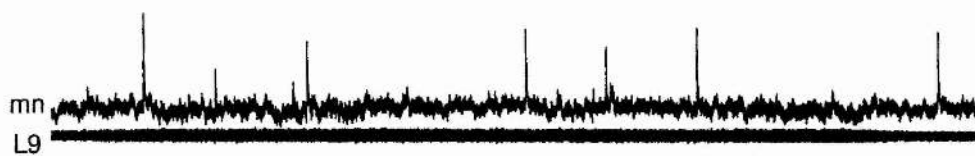
Figure 5.13. Synaptic drive to motoneurons during SNAP-induced motor activity.

A. Intracellular recording made with KCl-filled electrode from a presumed motoneurone (mn) located at the 4th post otic cleft. In control saline, motoneurons receive little phasic synaptic input and no discharge is seen to occur in the recorded ventral root cleft (L9) (i). 6 minutes after the bath application of 100 μ M SNAP, phasic depolarising potentials in motoneurons occur in phase with the discharge from the ventral roots. SNAP also induces a bout of rhythmic swimming activity in this example (asterisked in Aii).

B. Expanded trace of the activity boxed in Aii.

Ai

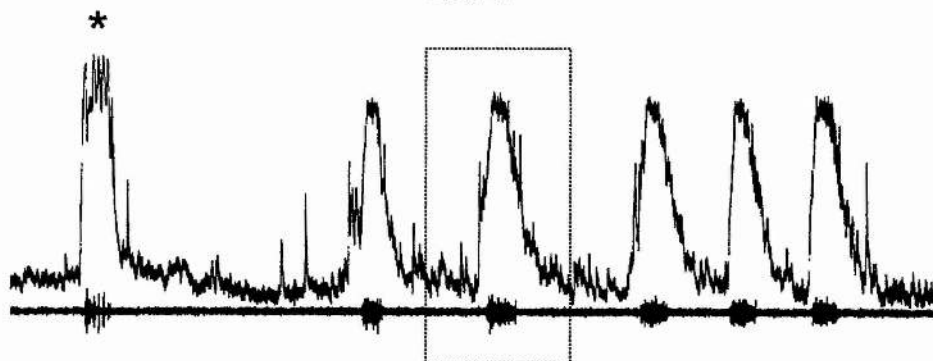
control



30mV
2sec

ii

SNAP



B

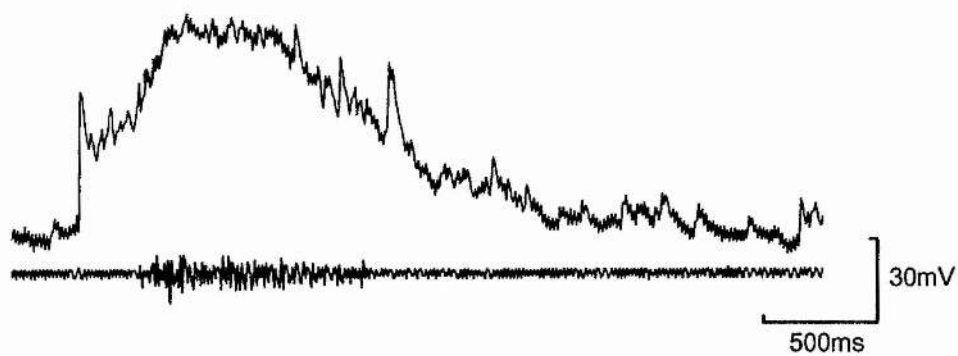
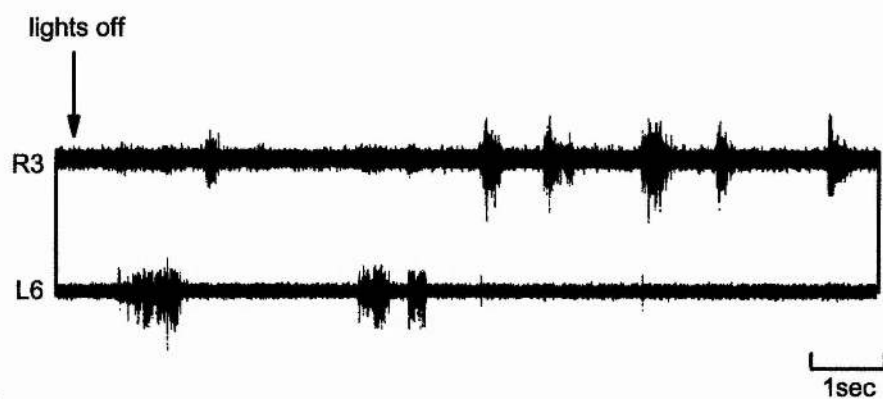


Figure 5.14. The motor pattern induced by SNAP resembles the response to dimming of illumination.

A. Ventral root recordings made from R3 and L6 after dimming of the illumination in control saline (i) and after 3.5 minutes exposure to SNAP under constant illumination (ii). Note the similarity in the ventral root response under each condition.

B. Intracellular recording made with a KCl-filled electrode from a motoneurone (mn) located at the level of the 5th post otic cleft after dimming of illumination in control saline (i) and after 5 minutes exposure to 100 μ M SNAP (ii) showing that the ventral root activity (L10) and the synaptic drive to motoneurones appears similar under each condition.

Ai

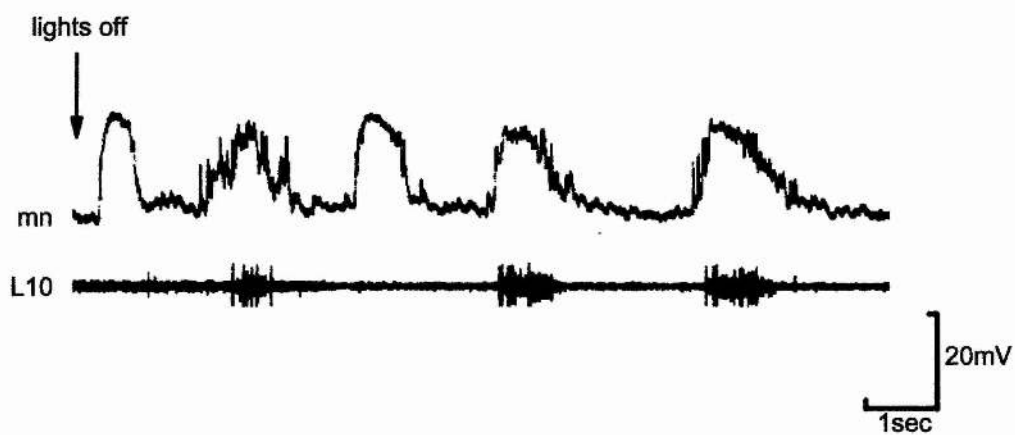


ii

SNAP



Bi



Bii

SNAP



figure 5.15. Under control conditions, no ventral root discharge upon light dimming (figure 5.15A). At around 5 minutes after addition of SNAP (100 μ M), several bursts of ventral root output are seen to occur in response to light dimming (figure 5.15B).

v) A possible role for NA in mediating the effects of NO.

The effects of SNAP clearly bear a very close resemblance to those of NA, suggesting a possible link between NA and NO in the initiation of motor output. For example NA might initiate locomotor output by triggering the endogenous liberation of NO, or vice versa. Alternatively, NA and NO could provide different routes for activation of the same motor circuitry. In order to study possible links between the two transmitter systems, the effects of NOS inhibitors and NA receptor antagonists on NA and SNAP-induced motor activity was studied.

Initial experiments examined the effects of the NOS inhibitor L-NOARG on NA-induced motor output. As shown in figure 5.16, animals which had been pre-incubated with 100-200 μ M L-NOARG were still capable of producing bouts of ventral root discharge upon exposure to NA (n=2). Where in control conditions (figure 5.16A) and after exposure to L-NOARG (figure 5.16B) there was no activity in the recorded motoneurons or ventral roots, following exposure to 10 μ M NA, in the presence of L-NOARG (100 μ M in this case) a motor program was initiated that was indistinguishable from that generated in the presence of NA alone (figure 5.16C). These findings would suggest that endogenous NO synthesis is not required for the effects produced by NA.

In order to ascertain whether the effects of SNAP are dependent on the presence of a functional noradrenergic system, phentolamine and alprenolol, which are broad spectrum α and β antagonists respectively were used to block NA

Figure 5.15. SNAP enhances the response to dimming of illumination in *Rana* embryos.

- A. Ventral root recordings made from R4 and L6 after dimming of the illumination in control conditions. Light dimming in this preparation does not induce any ventral root activity.
- B. Response to dimming of illumination in the same preparation as shown in A. 5 minutes after exposure to 100 μ M SNAP. Light dimming now initiates a period of ventral root activity 3 seconds after illumination is dimmed.

A

control

lights off



R4

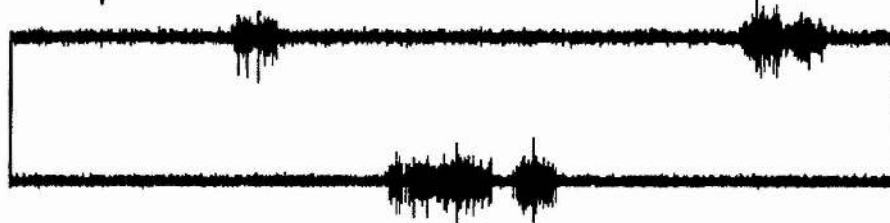
L6



B

SNAP

lights off



3sec

Figure 5.16. Effect of NOS inhibition on spontaneous activity induced by NA.

Intracellular recording made with a KCl-filled electrode from a motoneurone (mn) located at the level of the 6th post otic cleft in control saline (Ai) and 20 minutes after the bath application of the NOS inhibitor L-NOARG (100 μ M; B) and 4 minutes after subsequent application of 10 μ M NA (C).

A. During control conditions no phasic synaptic activity occurs in the recorded motoneurone. Ventral root activity (recorded from L11) is also absent.

B. 20 minutes after bath application of 100 μ M L-NOARG, no spontaneous activity is seen to occur.

C. 10 μ M NA induces phasic depolarising potentials in motoneurones after 4 minutes which are accompanied by bursts of discharge in the ventral root.

A

control



B

L-NOARG



3sec

C

L-NOARG + NA



receptors before subsequent bath application of SNAP. The results were somewhat inconclusive in that phentolamine was found to block the SNAP response in 2 of 5 preparations whereas alprenolol blocked the SNAP response in 3 of 5 preparations. An example of one of the experiments where alprenolol blocked the response to SNAP is shown in figure 5.17. No motor output occurs in control conditions (figure 5.17A) or after exposure to 30 μ M alprenolol (figure 5.17B) in this preparation. Subsequent addition of SNAP also fails to initiate a motor response (Figure 5.17C) whilst addition of SNAP after a 20 minute wash in control saline in the same preparation results in the generation of a motor response (figure 5.17D).

Figure 5.17. The response to SNAP can be blocked by alprenolol.

- A. Ventral root recordings made from the L3 and L11 show that in control saline, no motor activity occurs.
- B. After twenty minutes exposure to the β -antagonist alprenolol ($30\mu\text{M}$), the ventral roots remain silent.
- C. 6 minutes after addition of $100\mu\text{M}$ SNAP in the presence of alprenolol does not induce motor output.
- D. After a wash for 20 minutes in control saline, 4 minutes after re-application of $100\mu\text{M}$ SNAP bursts of ventral root discharge are induced.

A

control



B

alprenolol



2sec

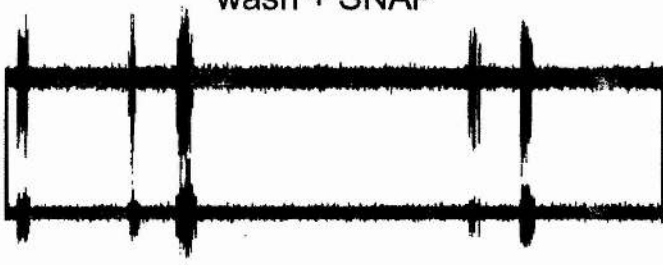
C

alprenolol + SNAP



D

wash + SNAP



DISCUSSION

i) Motor behaviour induced by NA and NO.

The results presented in this chapter provide evidence that the amine NA and the free radical gas, NO, can induce the same motor responses in embryos of the amphibian *Rana temporaria*. In both cases the activity is distinct from rhythmic swimming and is likely to underlie the strong body flexions that have previously been reported to occur in non-paralysed animals both spontaneously, in response to touch and following dimming of the illumination (Soffe, 1991a; Soffe & Sillar, 1991). The ventral root activity that characterises this response is coincident with phasic depolarisations in motoneurons, which are again similar to those previously reported by Soffe and Sillar (1991). Some depolarisations were not accompanied by ventral root discharge and these events are probably chloride-reversed inhibitory potentials arising from commissural interneurons on the opposite side of the spinal cord. I have also shown that in some cases, the motor response induced by both NA and SNAP could develop into brief episodes of rhythmic swimming. However, it should be noted that this effect was uncommon and invariably developed from an initial burst of non-rhythmic motor output. The non-rhythmic motor response was therefore the predominant response of NA and NO.

NA has previously been shown to initiate walking in the spinal cat, and my findings add weight to the suggestion that this amine is important for the induction of motor output in vertebrate motor systems. However, these results do not allow me to distinguish between whether NA is essential for initiating motor behaviour in *Rana* or whether it acts to enhance the systems responsiveness to other faster acting commands that in turn initiate motor activity. In the cat,

depletion of NA does not prevent initiation of locomotion by stimulation of the MLR (Steeves, Schmidt, Skovgaard & Jordan, 1980). In *Rana*, blockade of adrenergic receptors does not block swimming in response to skin stimulation, suggesting that NA is not essential for the generation of motor activity. The pharmacological characterisation of the response to NA is presently incomplete. Nonetheless, some success was achieved with both α and β receptor antagonists. The information currently available favours a predominant role for β receptors in mediating the effects of NA, although both types may play a role. It is clear that more data needs to be obtained before the mechanism of action of NA can be established.

The function of NO as a neuronal messenger was only recognised in the late 1980's, and since that time it has been shown to be released from endothelial cells, neurones, platelets, macrophages, neutrophils and smooth muscle cells. This small, gaseous molecule has been reported to be involved in a diverse array of CNS phenomenon (see introduction). With respect to rhythm generation, little is known of NO's effects. Until now the only report that NO is involved in motor control is from work done on the canine colon where NO induces an oscillatory motor pattern (Keef, Murray, Sanders & Smith, 1997). I have shown here that SNAP has a potent ability to activate spinal motor networks in *Rana* embryos. This discovery is of potentially great interest as I believe it is the first instance that NO has been implicated in control of spinal motor circuitry. Furthermore, the pattern of activity that occurs after the bath application of SNAP bares a remarkable similarity to that induced by NA: both agents cause spontaneous ventral root bursts that are accompanied by phasic depolarisations in motoneurones; both agents cause activity that propagates rostrocaudally but never occurs synchronously across the two sides of the spinal cord; the time-course for the onset and duration of NA and SNAP responses are also very similar. This raises the obvious question as to whether the two agents are acting

through two distinct mechanisms or whether there is convergence between their effects. In the vertebrate nervous system, NO can affect NA transmission in a variety of ways depending on the tissue and animal studied (see introduction). My findings that inhibition of NO synthesis with L-NOARG does not abolish the effect of NA indicates that NA is not initiating motor output by triggering synthesis of NO. However, applications of NA antagonists have achieved some success in blocking the response to SNAP and the information presented in this chapter suggests that if SNAP is inducing motor output by potentiating the presynaptic release of NA from nerve terminals, then the amine is likely to be acting at β -receptors. The pharmacology employed in this study unfortunately did not provide a conclusive answer, and I would not like to speculate further as to whether the two systems act through convergent pathways. It is clear that more pharmacological data needs to be obtained before any possible links between these two systems can be firmly established. Nevertheless, from the current literature, there is precedence to suggest that NO causes the presynaptic release of NA (see introduction). A modulation of NA release by NO may account for the initiation of motor output by both transmitters in *Rana* embryos.

ii) Possible behavioural relevance of the motor pattern induced by NA and NO.

By stage 20, the *Rana* embryo has developed the ability to express a range of motor behaviours (such as swimming and lashing), enabling it to survive its imminent free-living lifestyle. However, the non-rhythmic motor responses that occur at earlier stages can still be expressed. It is likely that all the different types of motor behaviour are generated by the same spinal networks. Therefore, the animal must have a means of re-configuring the network in order to select the

required behaviour. It has been shown in other motor systems, most notably the stomatogastric nervous system (see Harris-Warrick, Nagy & Nusbaum (1992)) that neuromodulators can alter the properties of a single neural network in such a way that enables it to generate several different motor behaviours. Perhaps NA and NO have a similar role in the *Rana* embryo. Aside from initiating motor activity, they may re-configure spinal motor networks to facilitate the expression of a particular form of behaviour from several possible configurations. This will allow the animal to initiate the appropriate motor response as dictated by environmental and developmental requirements. For example, NA and NO may be utilised for generation of non-rhythmic flexions whilst other transmitters may initiate other motor responses such as swimming and lashing.

What could the behavioural relevance of the non-rhythmic motor response be? Non-rhythmic body flexions are the earliest form of behaviour produced by this animal, occurring when it is still within its egg membrane (Soffe, 1991a). Perhaps NA and NO play an important role in development at this time. The motor pattern induced by NA and NO may be important for generating the appropriate response required to facilitate liberation from the egg membrane during hatching. In the chick embryo, hatching behaviour is characterised by back thrusts of the beak that are accompanied by short (1-2s) bursts of flexor-extensor activity in the hindlimbs. This particular form of motor behaviour is normally only expressed during the 45-90 minutes that is required for the animal to break free from its egg. Subsequently, other patterns of motor activity, such as walking, hopping and swimming, are expressed. However, hatching can be re-elicited in the post-embryonic chick by placing it in a glass egg (Beckoff & Kauer, 1984). Although stretch receptors in the neck appear to be important for triggering this response (Beckoff & Kauer, 1982; Beckoff & Sabichi, 1987), other signals, such as neurochemicals, have also been suggested to play a role (Beckoff & Kauer, 1982). Assuming that the response triggered by NA and NO in *Rana* embryos is

important for hatching, then they may serve a similar function in other animals such as the chick.

If the NA and NO-mediated motor pattern is important for hatching in the *Rana* embryo, then their role may be temporary during development. Early in development of the chick embryo, spinal flexor motoneurons receive GABAergic depolarising ipSPs during each half-cycle of motor activity. These potentials restrict the extent to which motoneurons can fire in each phase of activity. Later in development, the GABAergic inhibitory input is lost, an effect which coincides with the ability of motoneurons to fire in a different temporal sequence (O'Donovan, Sernagor, Sholomenko, Ho & Antal, 1992). A similar situation may occur in *Rana* embryos whereby the influence of NA and NO is removed from the motor network at later stages in development when the non-rhythmic motor response is no longer behaviourally relevant. Conversely, NA and NO could be conserved as effectors of the motor network but, due to new elements being introduced into the CPG and modifications of properties of pre-existing elements, their effects on motor output altered.

iii) Possible roles for NA and NO in generating responses to dimming of illumination.

The NA and NO mediated non-rhythmic motor pattern is very similar to that produced in response to dimming of illumination. The sensory pathways involved in generating the light dimming response are currently unknown. They are, however, unlikely to involve the lateral eyes as these are not fully developed at stage 20 and are still covered by a layer of pigmented skin. The pineal eye is a more likely candidate. The pineal eye of lower vertebrates functions to relay photic information to the brain. In the *Xenopus* tadpole, dimming of illumination

induces rhythmic swimming activity, a response that has been shown to be mediated through the pineal eye (Roberts, 1978; Foster & Roberts, 1982). It is possible that the light dimming response in *Rana* embryos is also generated by this brain structure. There is precedence to suggest that NA and NO systems are linked to pineal gland activity in mammals. The rat pineal gland has been shown to be innervated by NA-releasing postganglionic fibres that, when activated, cause elevations in cAMP and cGMP levels. NO appears to mediate the formation of cGMP in this system, as NO donors cause increased cGMP turnover. NOS inhibitors also decrease adrenergic stimulated cGMP production in this region of the brain, suggesting that NA induces NO synthesis in the rat pineal (Schaad, Vanecek, Kosar, Aubury & Schulz, 1995). On the basis of these observations it is possible that a similar process may generate the light dimming response in *Rana*. However, my results indicate that the response to NA is not affected in the presence of NOS inhibition. Such results are, however, preliminary and further investigations employing the use of a wider spectrum of NOS inhibitors need to be performed. Conversely, the results presented here suggest that NO could be inducing the release of NA which initiates motor output in response to light dimming. Further studies on the effects of adrenergic and NOS antagonism on the light dimming response will help to determine whether either transmitter is essential for generation of this behaviour. It may be that the pineal eye is not directly involved in the dimming response at all. Sensory pathways within the skin could putatively play a role. Indeed, the skin of *Rana* embryos contains NADPH-diaphorase (A.M. Woolston, personal communication) and is therefore a likely source of NO. Due to its gaseous nature, NO synthesis in the skin could have a direct influence on the CNS via simple diffusion. Furthermore, there is evidence that NO is generated upon exposure to light (Venturini, Palmer & Moncada, 1993). Therefore, light-mediated changes in NO production within the skin could

result in profound changes in NO levels within the CNS, which in turn may initiate motor activity.

iv) Comparison of the effects of NA in *Rana* and *Xenopus* preparations.

In the previous chapters I have shown that NA has a profound influence on swimming activity in *Xenopus* tadpoles, but does not initiate locomotion. This directly contrasts the situation in *Rana* embryos where NA initiates a non-rhythmic motor pattern, but does not modulate swimming activity. In chapter four, I proposed that the NA-induced modulation of motor output in *Xenopus* was a consequence of enhanced glycinergic transmission but the influence NA exerts on mid-cycle inhibition in *Rana* embryos has not yet been examined. Nonetheless, the amine appears to have very different actions in the different species. Studies on other vertebrate motor systems has shown the transmitters involved in generating motor output can be species specific (see introduction). Elements of the motor network in *Rana* embryos which may not be present in *Xenopus* embryos could mask the effects of NA during swimming making any modulation much more subtle. Indeed, swimming frequency, burst duration and longitudinal co-ordination are inherently more variable within a single episode of *Rana* swimming than they are in *Xenopus* tadpoles.

v) Final comments.

In summary, this chapter shows that both NA and NO can initiate similar non-rhythmic motor responses in the embryo of the frog *Rana temporaria*. The motor pattern induced by these agents comprises bursts of ventral root discharge

that are reminiscent of the non-rhythmic body flexions first described by Soffe (1991a). Both transmitters appear to cause the selective expression of this form of behaviour from a range of possible outputs, suggesting that different command systems can configure spinal motor networks to generate one particular type of behaviour. At this stage, it is not clear whether NO and NA systems are in some way physiologically linked, but this possibility cannot be excluded. Further experiments using pharmacological manipulations of these systems will help to clarify this point. The responses induced by both agents resemble the responses elicited by dimming of illumination. This suggests that NA and/or NO may indeed be liberated upon dimming of illumination and could be important for the generation of this behaviour.

CHAPTER 6

General discussion

The main goal of this study was to investigate the role of NA in the generation and modulation of motor activity in two simple models for vertebrate locomotion- *Xenopus* tadpoles and the *Rana* embryo. NA has profound but very different effects in both of these animals and my discoveries have raised some important issues with regard to the control of motor output by NA. In *Xenopus* tadpoles, one of the primary effects of the amine is to decrease swimming frequency. The ability of NA to slow rhythmic motor activities has already been documented in several other preparations including the lamprey (McPherson and Kemnitz, 1994) and cat (Barbeau and Rossignol, 1991) as well as the rhythm generating networks that control mammalian respiration (Errichidi, Hilaire and Monteau, 1990). Such reports, coupled with the findings I have presented here suggest the amines function may have been phylogenetically conserved during evolution, acting as a sort of physiological 'brake' for the neural networks that underlie locomotion. However, although NA has been shown to exert similar effects in the preparations outlined above, the mechanism of action through which it exerts these effects has remained unclear. During this study I have shown that NA strengthens spinal glycinergic synapses during swimming activity, an effect which may, at least in part, account for the effect the amine has on motor output. An increase in the amplitude of reciprocal glycinergic inhibition would presumably increase the duration of this phase of activity and therefore delay the onset of phasic excitation. This is, to my knowledge, the first time direct physiological evidence has been presented to account for the amines ability to reduce motor system rhythm frequency. Furthermore, my findings support the premise that the strength of reciprocal inhibition is not only a key target for neuromodulation, but is also important for the control of motor frequency (Dale, 1995b).

The strengthening of glycinergic inhibitory synapses in *Xenopus* appears to occur through a presynaptic mechanism, as NA increases the occurrence of

spontaneous ipsp's in the presence of TTX. The concept that neuromodulators regulate transmitter release from presynaptic terminals is now widely accepted. (e.g. Mintz Gotow, Triller and Korn, 1989; Wall and Dale, 1993; Shupliakov, Pierbone, Gad and Brodin, 1995). Of direct relevance to this study is that NA specifically enhances synaptic transmission via a presynaptic mechanism in several other preparations. For example, in cerebellar stellate and Purkinje cells, the amine enhances the frequency of occurrence of GABAergic spontaneous (s) and miniature (m) inhibitory post synaptic currents (ipscs) without an effect on amplitudes or kinetics of these currents (Llano and Gerschenfeld, 1993; Kondo and Marty, 1997). This effect is similar to that seen in CA1 hippocampal neurones where the frequency but not amplitude of miniature excitatory post synaptic currents (epscs) is increased by NA (Gereau and Conn, 1994). The NA-mediated effects in the cerebellar synapses have a β -adrenergic receptor profile and can be mimicked by forskolin, a potent activator of adenylyl cyclase whilst in cells located in the CA1 region, H89 (an inhibitor of PKA) blocks the response to NA. Furthermore the increase in (m)ipscs in stellate cells is blocked by Rp-cAMPS, a potent inhibitor of cAMP dependent protein kinase (PKA). Such findings implicate cAMP-dependent second messenger pathways in the noradrenergic modulation of synaptic strengths, an effect which is likely to be mediated through β -receptors as they are known to be positively coupled to adenylyl cyclase. My findings also indicate that NA receptors exist on inhibitory interneurons that presynaptically regulate the release of GABA and glycine. It is not yet clear whether this effect is also mediated by cAMP dependent kinases or via other mechanisms. The extracellular studies presented here show that α -adrenoceptors appear to mediate the effects of NA effects on swimming behaviour although it remains to be established whether the effects on inhibitory synapses occur through the same physiological mechanisms. Pathways other than those that stimulate adenylyl cyclase may well be involved: NA enhances the amplitude of excitatory

post synaptic currents (epscs) in the chick ciliary presynaptic terminal, an effect which is thought to be due to an enhancement in the presynaptic sensitivity of the exocytotic machinery to Ca^{2+} (Yawo, 1996). This response appears to be mediated via a pharmacologically distinct receptor subtype. Future experiments in *Xenopus* tadpoles using agonists and antagonists for the different classes of NA receptor as well as pharmacological manipulation of second-messenger pathways will help to identify the precise mechanism through which NA affects inhibitory transmission in the spinal cord of *Xenopus* tadpoles. One final point that requires attention with regard to this matter is that NA-induced enhancements of spontaneous and evoked glycine release in *Xenopus* could be mediated through two distinct and unconnected pathways. In the rat cerebellar stellate cells, NA increases the probability of spontaneous inhibitory transmitter release in the presence of TTX (Kondo & Marty, 1997). However, experiments performed in the absence of sodium channel blockade show that NA decreases the probability of evoked release (Kondo & Marty, 1998). This suggests that action potential-dependent and action potential-independent release mechanisms are regulated independently of each other (Kondo & Marty, 1998). Nonetheless, my findings show that NA both increases the amplitude of reciprocal glycinergic inhibition and also increases the probability of spontaneous glycine release in the presence of TTX. It remains to be seen if the effect on evoked release reflects an increase probability of spontaneous release.

The possible role of NA in the development of motor behaviour needs to be addressed. Between embryonic stage 37/8 and larval stage 42 rapid developmental changes occur in the animal. At stage 37/8 the animal spends around 98% of its life static, attached to various surfaces in the water via a strand of mucus secreted from the cement gland. Its food source is derived entirely from the yolk sack and as such the animal has little requirement for a flexible motor system. By stage 42, just 24h later, the animal is around 40% larger, the yolk sack

has largely been consumed, the mouth and gut have developed and the tadpole is close to reaching an extended period of free-swimming, free-feeding larval life. As such, during this period the motor capability of the animal does become much more complex, an effect which is coincident with the innervation of the spinal cord by brain stem interneurone axons. The appearance of axons that descend into the spinal cord at this stage is thought to be causal in imparting the newly acquired motor flexibility of the stage 42 larvae (vanMier, Joosten, van Reden & ten Donkelaar, 1986; Sillar, Woolston & Wedderburn, 1995). One such brainstem modulator is 5-HT, arising from raphe neurones which invade to spinal cord during this period of development and profoundly modulate motor output (see chapter 1). This present study has examined a possible role for NA in the development of a more complex and flexible motor pattern. However, as NA appears to affect motor output in very similar ways between the two stages, my results do not allow me to establish whether a functional descending noradrenergic influence is already present by stage 37/8, but they do suggest that NA receptors can be found within the spinal cord as early as stage 37/8. It is clear that immunocytochemical studies now need to be performed in order to establish when noradrenergic input to the spinal cord arises in this animal.

The effects of NA on *Xenopus* embryo swimming at both the synaptic and behavioural level contrast with those produced by 5-HT. NA increases mid-cycle ipsp amplitudes whilst 5-HT decreases them. The modulation of the swimming motor pattern induced by the amines is also very different. NA slows down swimming and decreases delays whilst 5-HT intensifies ventral root output and strengthens the relationship between delays and cycle periods. Both amines therefore cause the expression of different forms of swimming generated from, presumably, the same CPG. This ability to produce a range of behaviours from the same neural network is an essential means of imparting flexibility to the motor system. NA could be required to 'reverse' or counteract the effects of 5-HT on the

motor pattern. In such a way, NA may help to maximise the range of outputs that the motor network is capable of, making it dynamic and able to adjust to any given contingency.

In *Rana* embryos, I have shown that NA has little effect on fictive swimming behaviour but instead initiates a pattern of non-rhythmic motor output. The ability of NA to activate CPGs for motor generation has been documented in the cat (Kiehn, Hultborn & Conway, 1992), although similar effects have not been reported in other vertebrates. The motor pattern induced by NA in *Rana* embryos is also produced by the nitric oxide donor SNAP. Very little is known of the influence NO exerts on rhythm-generating networks, and I believe this is one of the first accounts to suggest a role for this gaseous messenger in motor control. It is not clear as to whether the two neuronal messengers generate motor behaviour through the same mechanism, but this possibility cannot be excluded (see chapter 5 discussion). The fact that NA and NO induce behaviour that is remarkably similar to that which occurs in response to dimming of the illumination in these animals is of potential interest. In chapter 5, I discussed the possible role of the pineal eye in generation of this response, with reference to the possible involvement of NA and NO. In amphibian embryos, the pineal eye serves as a direct photosensor for the CNS, but this is not the case in higher vertebrates. In mammals, the pineal gland plays a different role in that it regulates sleep/wake cycles by secretion of the hormone melatonin. The mammalian pineal gland is not thought to be photosensitive. Instead, a small cluster of cell bodies called the suprachiasmatic nucleus (SCN) is responsible for relaying photic information received by the visual system in mammals. This tiny structure regulates the circadian clock via light-sensitive adjustments in circadian oscillations. In mammals the activity of the SCN is controlled by noradrenergic and NO systems. Exposure to light causes release of NO which induces phase shifts in the timing of these rhythms (Ding, Chen, Weber, Fairman, Rea and Gillette, 1994). There is

precedence to suggest that synthesis of NO in the pineal gland is regulated by noradrenergic input, so that NOS activity is induced by NA (Schaad, Vanecek, Kosar, Aubry, Schulz, 1995). Furthermore, the activity of pineal gland (which receives input from the SCN), and therefore the release of melatonin is also regulated by NA and NO (see discussion in chapter 5). On the basis of these reports, it is now becoming apparent that the amine NA and the gaseous neural messenger NO play an important role in regulation of photosensitive biological processes, and my findings may support this premise.

References

- Addicks, K., Bloch, W. & Feelisch, M. (1994) Nitric-oxide modulates sympathetic neurotransmission at the prejunctional level. *Microscopy Res. Tech.* **29**, 161-168.
- Ader, J.P., Postema, F. & Korf, J. (1979) Contribution of the locus coeruleus to the noradrenergic innervation of the rat spinal cord: a biochemical study. *J. Neural Trans.* **44**, 159-173.
- Ahlquist, R.P. (1948) A study of adrenotropic receptors. *Am. J. Physiol.*, **153**, 586-600.
- Alford, S. & Williams, T.L. (1987) Evidence that glycine mediates left-right alternation in the lamprey spinal cord. *J. Physiol. Lond.*, **390**, 36P.
- Alford, S. & Williams, T.L. (1989) Endogenous activation of glycine- and NMDA receptors in the lamprey spinal cord during fictive locomotion. *J. Neurosci.*, **9**, 2792-2800.
- Alford, S. & Grillner, S. (1990) CNQX and DNQX block non-NMDA synaptic transmission but not NMDA evoked locomotion in lamprey spinal cord.
- Alford, S., Christenson, J. & Grillner, S. (1990) Presynaptic GABA_A and GABA_B receptor-mediated phasic modulation in axons of spinal motor interneurons. *Eur. J. Neurosci.* **3**, 107-117.

Alford, S. Sigvardt, K.A. & Williams, T.L. (1990) GABAergic control of rhythmic activity in the presence of strychnine in the lamprey spinal-cord. *Brain Res.* **506**, 303-306.

Andén, N.E., Jukes, M.G.M., Lundberg, A. & Vyklicky, L. (1966) The effect of L-DOPA on the spinal cord. I. Influence on transmission from primary afferents. *Acta. Physiol. Scand.* **67**, 373-386.

Aprinson, M.H. (1990) The discovery of the transmitter role of glycine. In: *Glycine Neurotransmission*. Edited by: O.P. Otterson and J. Storm-Mathisen. Chinchester, UK. 1-23.

Bacq, Z.M. (1934) La pharmacologie du systeme nerveux autonome, et particulierement du sympathetique, d'apres la theorie neurohumorale. *Annales du physiologie et de physicochimie biologique*, **10**, 467-528.

Baltensperger, K., Chiesi, M. & Carafoli, E. (1990) Substrates of cGMP kinase in vascular smooth-muscle and their role in the relaxation process. *Biochemistry*, **29**, 9753-9760.

Barasi, B. & Roberts, M.H.T. (1977) Responses of motoneurones to electrophoretically applied dopamine. *Br. J. Pharmacol.* **60**, 29-34.

Barbeau, H. & Rossignol, S. (1989) Effects of noradrenergic, serotonergic and dopaminergic drugs on the initiation of locomotion in the adult spinal cat. *Soc. Neurosci. Abs.* **15**, 393.

Barbeau, H. & Rossignol, S. (1991) Initiation and modulation of the locomotor pattern in the adult chronic spinal cat by noradrenergic, serotonergic and dopaminergic drugs. *546*, 250-260.

Barbeau, H., Julien, C. & Rossignol, S. (1987) The effects of clonidine and yohimbine on locomotion and cutaneous reflexes in the adult chronic spinal cat. *Brain Res.*, **437**, 83-96.

Barbeau, H., Chau, C. & Rossignol, S. (1993) Noradrenergic agonists and locomotor training affect locomotor recovery after cord transection in adult cats. *Brain Res. Bull.*, **30**, 387-393.

Barker, R.G. & Anderson, E.G. (1970) The effect of L-3,4-dihydroxyphenylalanine on spinal reflex activity. *J. Pharmacol. Exp. Ther.* **173**, 212-223.

Barry, M.J. & O'Donovan, M.J. (1987) The effects of excitatory amino acids and their antagonists on the generation of motor activity in the isolated chick spinal cord. *Dev. Brain Res.* **36**, 271-276.

Batty, R.S. (1984) Development of swimming movements and musculature of larval herring (*Clupea-harengus*). *J. Exp. Biol.*, **110**, 217.

Beckoff, A. (1992) Neuroethological approaches to the study of motor development in chicks: Achievements and Challenges. *J. Neurobiol.*, **23**, 1486-1505.

Beckoff, A. & Kauer, J.A. (1982) Neural control of hatching: Role of neck position in turning on hatching leg movements in post-hatching chicks. *J. Comp. Physiol.* **145**, 497-504.

Beckoff, A. & Kauer, J.A. (1984) Neural control of hatching: Fate of the pattern generator for the leg movements of hatching in posthatching chicks. *J. Neurosci.* **4**, 2659-2666.

Beckoff, A. & Sabichi, A. L. (1987) Sensory control of the initiation of hatching in chicks: Effects of a local anaesthetic injected into the neck. *Dev. Psychobiol.* **20**, 489-495.

Belcher, G., Ryall, R.W. & Schaffner, R. (1978) The differential effects of 5-hydroxytryptamine, noradrenaline and raphe stimulation on nociceptive and non-nociceptive dorsal horn interneurons in the cat. *Brain Res.* **151**, 307-321.

Biel, M., Altenhofen, W., Hullin, R., Ludwig, J., Freichel, M., Flockerzi, V., Dascal, N., Kaupp, U.B. & Hofmann, F. (1993) Primary structure and functional expression of a cyclic nucleotide gated channel from rabbit aorta. *Febs Lett.* **329**, 134-138.

Biscoe, T.J., Curtis, D.R. & Ryall, R.W. (1966) An investigation of catecholamine receptors of spinal interneurons. *Int. J. Neuropharmacol.* **5**, 429-434.

Björklund, A. & Skagerberg, T. (1982) Descending monoaminergic projections to the spinal cord. In: *Brainstem control of spinal mechanisms*. Ed. by B. Sjölund & A. Björklund. Elsevier Bio-Medical, Amsterdam, pp. 55-88.

Bohme, G.A., Bon, C., Stutzmann, J.M, Doble, A. & Blanchard, J.C. (1991) Possible involvement of nitric-oxide in long-term potentiation. *Eur. J. Pharmacol.* **199**, 379-381.

Boothby, K.M. & Roberts, A. (1992) The stopping response of *Xenopus laevis* embryos: behaviour, development and physiology. *J. Comp. Physiol.* **170A**, 171-180.

Brodin, L. & Grillner, S. & Rovainen, C.M. (1985) N-methyl-D-aspartate (NMDA), kainate and quisqualate receptors and the generation of fictive locomotion in the lamprey spinal cord. *Brain Res.* **325**, 302-306.

Brown, T.G. (1911) The intrinsic factor in the act of progression in the mammal. *Proc. Roy. Soc. Lond. B.* **84**, 308-319.

Brüning, G. & Mayer, B. (1996) Localization of nitric oxide synthase in the brain of the frog, *Xenopus laevis*. *Brain Res.* **741**, 331-343.

Buchanan, J. T. (1982) Identification of interneurons with contralateral caudal axons in the lamprey spinal cord: synaptic interactions and morphology. *J. Neurophysiol.*, **47**, 961-975.

Buchanan, J.T. (1996a) Lamprey spinal interneurons and their roles in swimming activity. *Brain Behav. Evol.* **48**, 287-296.

Buchanan, J.T. (1996b) The neuronal network for locomotion in the lamprey spinal-cord -evidence for the involvement of commissural interneurons. *J. Physiol. (Paris)*. **89**, 221-233

Buchanan, J.T. & Cohen, A. H. (1982) Activities of identified interneurons, motoneurons, and muscle fibres during fictive swimming in the lamprey and effects of reticulospinal and dorsal cell stimulation. *J. Neurophysiol.*, **47**, 948-960.

Buchanan, J.T. & Grillner, S. (1987) Newly identified 'glutamate interneurons' and their role in locomotion in the lamprey spinal cord. *Science*, **236**, 312-314.

Buchanan, J.T. & Grillner, S. (1988) A new class of small inhibitory interneurons in the lamprey spinal cord. *Brain Res.* **438**, 404-407.

Calvillo, O. & Ghignone, M. (1986) Presynaptic effect of clonidine on unmyelinated afferent-fibers in the spinal-cord of the cat. *Neurosci. Lett.*, **64**, 335-339.

Carlsson, A., Dahlström, A., Fuxe, K. & Hillarp, H. (1964) A cellular localisation of monoamines in the spinal cord. (1964) **60**, 112-119.

Carstens, E., Gilly, H., Schreiber, H. & Zimmermann, M. (1987) Effects of midbrain stimulation and iontophoretic application of serotonin, noradrenaline, morphine and GABA on electrical of afferent c-fiber and a-fiber terminals in cat spinal-cord. *Neuroscience*, **21**, 395-406.

Cazalets, J.R., Grillner, P., Menard, I., Cremieux, J. & Clarac, F. (1990) 2 types of motor rhythm induced by NMDA and amines in an in vitro spinal-cord preparation of neonatal rat. *Neurosci. Lett.* **111**, 116-121.

Cazalets, J.R., Sqalli-Houssaini, Y. & Clarac, F. (1992) Activation of the central pattern generators for locomotion by serotonin and excitatory amino-acids in neonatal rat. *J. Physiol. (Lond)*. **455**, 187-204.

Cazalets, J. R., Sqalli-Houssaini, Y. & Clarac, F. (1994) GABAergic inactivation of the central pattern generator for locomotion in isolated neonatal rat spinal cord. *J. Physiol.* **474**, 173-181.

Clark, F.M. & Proudfit, H.K. (1993) Group to the spinal-cord in the rat - anatomical evidence that a5-neurons modulate nociception. *B. Res.* **616**, 200-210.

Clarke, J.D.W. & Roberts, A. (1984) Interneurones in the *Xenopus* embryo spinal cord: sensory excitation and activity during swimming. *J. Physiol. (Lond)*. **354**, 345-362.

Clarke, J.D.W., Hayes, B.P., Hunt, S.P. & Roberts, A. (1984) Sensory physiology, anatomy and immunohistochemistry of Rohon-Beard neurones in embryos of *Xenopus laevis*.

Cohen, A. H. & Wallén, P. (1980) The neural correlate of locomotion in fish. 'Fictive swimming' induced in an in vitro preparation of the lamprey spinal cord. *Exp. Brain Res.*, **41**, 11-18.

Cohen, A. H. & Harris-Warrick, R. M. (1984) Strychnine eliminates alternating motor output during fictive locomotion in the lamprey. *Brain Res.*, **293**, 164-167.

Cohen A.H., Tamara, A., Dobrov, G.L., Keimel, T. & Baker, M.T. (1990) The development of the lamprey pattern generator for locomotion *J. Neurobiol.*, **21**, 958-969.

Connel, L.A., Majid, A. & Wallis, D.I. (1989) Involvement of $\alpha 1$ adrenoceptors in the depolarising but not the hyperpolarising responses of motoneurons in the neonate rat to noradrenaline. *Neuropharmacol.* **122**, 387-392.

Cowley, K.C. & Schmidt, B.J. (1994) A comparison of motor patterns induced by N-methyl-D-aspartate, acetylcholine and serotonin in the in vitro neonatal rat spinal cord. *Neurosci. Lett.* **171**, 147-150.

Cowley, K.C. & Schmidt, B.J. (1995) Effects of inhibitory amino acids antagonists on reciprocal inhibitory interactions during rhythmic motor activity in the in vitro neonatal rat spinal cord. *J. Neurophysiol.* **74**, 1109-1117.

Cruce, W.L.R., Stuesse, S.L. & Northcutt, R.G. (1992) Immunohistochemical localisation of catecholaminergic cells in cartilaginous fishes. *Abstr. 7th Intern. Catechol. Symp. Amsterdam.* p.60.

Dahlström, A. & Fuxe, K. (1964) Evidence for the existence of monoamine neurones in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurones. *Acta Physiol. Scand.* **62**, 1-55.

Dahlström, A. & Fuxe, K. (1965) Evidence for the existence of monoamine neurones in the central nervous system. II. Experimentally induced changes in interneuronal amine levels in the bulbospinal neuron systems. *Acta Physiol. Scand.* **64**, 1-36.

Dale, N. (1985) Reciprocal inhibitory interneurons in the *Xenopus* embryo spinal cord. *J. Physiol.* **363**, 61-70.

Dale, N. (1995a) Kinetic characterization of the voltage-gated currents possessed by *Xenopus* embryo spinal neurones. *J. Physiol.* **489**, 473-488.

Dale, N. (1995b) Experimentally derived model for the locomotor pattern generator in the *Xenopus* embryo. *J. Physiol.* **489**, 489-510.

Dale, N. & Roberts, A. (1984) Excitatory amino acid receptors in *Xenopus* embryo spinal cord and their role in the activation of swimming. *J. Physiol.* **348**, 527-543.

Dale, N. & Roberts, A. (1985) Dual-component amino acid-mediated synaptic potentials: excitatory drive for swimming in *Xenopus* embryos. *J. Physiol.* **363**, 35-59.

Dale, N.C. & Soffe, S.R. (1991) Kinematic analysis of amphibian tadpole swimming. *J. Mar. Biol. Assoc. (Lond.)* **71**, 734.

Dale, N., Otterson, O.P., Roberts, A. & Storm-Mathisen, J. (1986) Inhibitory neurons of a motor pattern generator in *Xenopus* revealed by antibodies to glycine. *Nature*. **324**, 255-257

Dale, N., Roberts, A. Ottersen, O.P. & Storm-Mathisen, J. (1987). A class of cerebrospinal fluid contacting neurones revealed in the frog embryo spinal cord by GABA immunocytochemistry. *Proc. Roy. Soc. Lond. B.*, **232**, 193.

Dawson, V.L., Dawson, T.M., London, E.D., Bredt, D.S. & Snyder, S.H. (1991) Nitric-oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Nat. Acad. Sci. USA.* **88**, 6368-6371.

Desai, K.M., Sessa, W.C. & Vane, J.R. (1991) Involvement of nitric-oxide in the reflex relaxation of the stomach to accommodate food or fluid. *Nature.* **351**, 477-479.

De Vries, J.I.P., Visser, G.H.A. & Prechtl, H.F.R. (1984) Foetal motility in the first half of pregnancy. In *Continuity of neural functions from prenatal to postnatal life. Clinics in Developmental Medicine.* No. 94. Edited by Prechtl, H.F.R., Oxford: Spastics International Medical Publications. 46-64.

Dickenson, P. (1989) Modulation of simple motor patterns. *Seminars in Neurosci.* **1**, 15-24.

Ding, J.M., Chen, D., Weber, E.T., Fairman, L.E., Rea, M.A. & Gillette, M.U. (1994) Resetting the biological clock -mediation of nocturnal shifts by glutamate and NO. *Science.* **266**, 1713-1717.

Douglas, J.R., Noga, B.R., Dai, X. & Jordan, L.M. (1993) The effects of interathecal administration of excitatory amino acid agonists and antagonists on the initiation of locomotion in the adult cat. *J. Neurosci.* **13**, 990-1000.

Dubuc, R., Cabelguen, J.M. & Rossignol, S. (1988) Rhythmic fluctuation of dorsal root potentials and antidromic discharges of primary afferents during fictive locomotion in the cat. *J. Neurophysiol.* **60**, 2014-2036.

Ekström, P.T., Reschke, H.W.M., Steinbusch & Th. van Venn (1986) Distribution of noradrenaline in the brain of the teleost *Gastreroosteus aculeatus*. L. An immunohistochemical analysis. *J. Comp. Neurol.* **254**, 297-313.

Elliot, T.R. (1904) On the action of adrenalin. *J. Physiol. (Lond)*. **32**, 401-467.

Engberg, I. & Marshall, K.C. (1971) Mechanism of noradrenaline hyperpolarisation in spinal cord motoneurons of the cat. *Acta. Physiol. Scand.* **83**, 142-144.

Engberg, I. & Ryall, R.W. (1966) The inhibitory action of noradrenaline and other monoamines on spinal neurones. *J. Physiol. (Lond)*. **185**, 298-232.

Engberg, I., Flatman, J.A. & Kadzielawa, K. (1976) Lack of specificity of motoneurone responses to microiontophoretically applied phenolic amines. *Acta Physiol. Scand.* **96**, 137-139.

Errichidi, S., Hilaire, G. & Monteau, R. (1990) Permanent release of noradrenaline modulates respiratory frequency in the newborn rat: an *in vitro* study. *J. Physiol. (Lond)*. **429**, 497-510.

Evans, R.H. & Watkins, J.C. (1978) Specific antagonism of the excitant amino acids in the isolated spinal cord of the neonatal rat. *Eur. J. Pharmacol.* **50**, 123-129.

Falk, B., Hillarp, N.A., Thieme, G. & Torp, A. (1962) Fluorescence of catecholamines and related compounds with formaldehyde. *J. Histochem. Cytochem.* **10**, 348-354.

Fenau, F., Corio, M., Palisses, R. & Viala, D. (1991) Effects of a NMDA-receptor antagonist, MK-801, on central locomotor programming in the rabbit. *Exp. Brain Res.* **86**, 392-401.

Fesenko, E.E., Kolesnikov, S.S. & Lyubarsky, A.L. (1985) Induction by cyclic-GMP of cationic conductance in plasma-membrane of retinal rod outer segment. *Nature*, **313**, 310-313.

Fleetwood-Walker, S.M., Mitchell, R., Hope, P.J., Molony, V. & Iggo, A. (1985) An α -2 receptor mediates the selective-inhibition by noradrenaline of nociceptive responses of identified dorsal horn neurons. *Brain Res.* **334**, 243-254.

Fossberg, H. & Grillner, S. (1973) The locomotion of the acute spinal cat injected with clonidine i.v. *Brain Res.* **50**, 184-186.

Foster, R.G. & Roberts, A. (1982) The pineal eye in *Xenopus* embryos and larvae: A photoreceptor with direct excitatory effects on behaviour. *J. Comp. Physiol.* **145**, 413-419.

Fritschy, J.M. & Grzanna, R. (1990) Demonstration of two descending noradrenergic pathways to the rat spinal cord: evidence for an intragrainal trajectory of locus coeruleus axons in the superficial layers of the dorsal horn. *J. Comp. Neurol.* **291**, 553-582.

Fritschy, J.M., Lyons, W.E., Mullen, C.A., Kosofsky, B.E., Molliver, M.E. & Grzanna, R. (1987) Distribution of locus coeruleus axons in the rat spinal cord: a combined anterograde transport and immunohistochemical study. *Brain Res.* **437**, 176-180.

Fung, S. & Barnes, C.D. (1981) Evidence of facilitatory coeruleospinal action in lumbar motoneurons of cats. *Brain Res.* **216**, 299-311.

Furchgott, R.F. & Zawadzki, J.V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 385-388.

Fuxe, K. (1965) Evidence for the existence of monoamine neurones in the central nervous system. IV. The distribution of monoamine terminals in the central nervous system. *Acta. Physiol. Scand.* **247**, 39-85.

Gallagher, B.C. & Moody, S.A. (1987) Development of substance P-like immunoreactivity in *Xenopus* embryos. *J. Comp. Neurol.* **260**, 175-185.

Gereau, R.W. IV & Conn, J. (1994) Presynaptic enhancement of excitatory synaptic transmission by β -adrenergic receptor activation. *J. Neurophysiol.* **72**, 1438-1442.

Gonzalez, A. & Smeets W.J.A.J. (1993) Noradrenaline in the brain of the South-African clawed frog *Xenopus laevis* -a study with antibodies against noradrenaline and dopamine-beta-hydroxylase. *J. Comp. Neurol.* **331**, 363-374.

Gonzalez, A. & Smeets, W.J.A.J. (1995) Noradrenergic and adrenergic systems in the brain of the urodele amphibian, *Pleurodeles waltlii*, as revealed by immunohistochemical methods. *Cell And Tissue Research.* **279**, 619-627.

Gonzalez, A., Marin, O., Tuinhof, R. & Smeets, W.J.A.J. (1994) Ontogeny of catecholamine systems in the central-nervous-system anuran amphibians -an immunohistochemical study with antibodies against tyrosine-hydroxylase and dopamine. *J. Comp. Neurol.* **346**, 63-79.

Gosner, K.L. (1960) A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica.* **16**, 183-190.

Green, C.S. & Soffe, S.R. (1996) Transition between two different motor patterns in *Xenopus* embryos. *J.Comp. Physiol.* **178**, 279-291.

Green, C.S. & Soffe, S.R. (1998) Roles of ascending inhibition during two rhythmic motor patterns in *Xenopus* tadpoles. *J.Neurophysiol.* **79**, 2316-2328.

Grillner, S. & Matsushima, T. (1991) The neural network underlying locomotion in the lamprey- synaptic and cellular mechanisms. *Neuron.* **7**, 1-15.

Grillner, S. & Wallén, P. (1980) Does the central pattern generation for locomotion in the lamprey depend on glycine inhibition? *Acta. Physiol. Scand.* **110**, 103-105.

Grillner, S. & Zangger, P. (1979) On the central generation of locomotion in the low spinal cat. *Exp. Brain Res.* **34**, 241-261.

Grillner, S., McClellan, A., Sigvardt, K., Wallén, P. & Wilen, M. (1981) Activation of NMDA-receptors elicits 'fictive locomotion' in lamprey spinal cord *in vitro*. *Acta. Physiol. Scand.* **113**, 549-551.

Grossman, W., Juma, I. & Nell, T. (1975) The effect of reserpine and DOPA on reflex activity in the rat spinal cord. *Exp. Brain Res.* **22**, 351-361.

Hagevik, A. & McClellan, A.D. (1994) Coupling of spinal locomotor networks in larval lamprey revealed by receptor blockers for inhibitory amino-acids - Neurophysiology and computer modelling. *J. Neurophysiol.* **72**, 1810-1829.

Haley, J.E., Wilcox, G.L. & Chapman, P.F. (1992) The role of nitric-oxide in hippocampal long-term potentiation. *Neuron*. **8**, 211-216.

Harris-Warrick, R.M. & Marder, E. (1991) Modulation of neural networks for behaviour. *Ann. Rev. Neurosci.* **14**, 39-57.

Harris-Warrick, R.M., Nagy, F. & Nusbaum, M.P. (1992) in *Dynamic biological networks: The stomatogastric nervous system*. Edited by Ronald M. Harris-Warrick, Eve Marder, Allen I. Selverston and Maurice Moulins. Bradford Press. pp 87-137.

Headley, P.M., Duggan, A.W. & Greirsmith, B.T. (1978) Selective reduction by nordrenaline and 5-hydroxytryptamine of nociceptive responses of cat dorsal horn neurones. *J.Physiol.* **145**, 185-189.

Hernandez, P., Elbert, K. & Droge, M. H. (1991) Spontaneous and NMDA evoked motor rhythms in the neonatal mouse spinal cord: an *in vitro* study in comparison with *in situ* activity. *Exp. Brain Res.* **85**, 66-74.

Hino, M., Ono, H. & Fukuda, H. (1987) Involvement of noradrenergic systems in the effects of conditioning stimulation of the lower brain-stem on the lumbar spinal reflex in rats. *Gen. Pharmacol.* **18**, 41-45.

Ho, S.M. (1997) Rhythmic motor activity and interlimb co-ordination in the developing pouch of the young wallaby (*Macropus eugenii*). *J. Physiol. (Lond)*. **501**, 623-636.

Hökfelt, T., Elfvin, L.G., Elde, R., Schultzberg, M., Goldstein, M. & Luff, R. (1977) Occurrence of somatostatin -like immunoreactivity in some peripheral sympathetic noradrenergic neurones. *Proc. Nat. Acad. Sci. USA.* **74**, 3587-3591.

Hornby, P.J. & Piekut, D.T. (1988) Immunoreactive dopamine- β -hydroxylase in neuronal groups in the goldfish brain. *Brain Behav. Evol.* **32**, 252-256.

Howe, J.R. & Zieglgänsberger, W. (1987) Responses of rat dorsal horn neurons to natural stimulation and to iontophoretically applied norepinephrine. *J. Comp. Neurol.* **255**, 1-17.

Hughes, A.F.W. (1957) The development of the primary sensory system in *Xenopus laevis* spinal cord. *J. Anat.* **91**, 323-338.

Hultborn, H. Petersen, N., Brownstone, R. & Nielsen, J. (1993) Evidence of fictive spinal locomotion in the marmoset (*callithrix Jacchus*). *Soc. Neurosci. Abstr.* **19**, 539.

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E. & Chaudhuri, G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric-oxide. *Proc. Nat. Acad. Sci. US.* **84**, 9265-9269.

Jacobsen, M. & Huang, S. (1985) Neurite outgrowth traced by means of horse radish peroxidase inherited from neuronal ancestral cells in frog embryos. *Dev. Biol.* **110**, 102-113.

Janowska, E., Jukes, M.G., Lund, S. & Lundberg, A. (1967a) The effect of DOPA on the spinal cord. 5. Reciprocal organisation of pathways transmitting excitatory action on alpha motoneurons of flexors and extensors. *Acta Physiol. Scand.* **70**, 369-388.

Janowska, E., Jukes, M.G., Lund, S. & Lundberg, A. (1967b) The effect of DOPA on the spinal cord. 6. Half-centre organisation of interneurons transmitting effects from the flexor reflex afferents. *Acta Physiol. Scand.* **70**, 389-402.

Jeftinija, S., Semba, K. & Randic, M. (1981) Norepinephrine reduces excitability of single cutaneous primary afferent c-fibers in the cat spinal-cord. *B. Res.* **219**, 456-463.

Jordan, L.M. (1983) Factors determining motoneurone rhythmicity during fictive locomotion. in *Neural origins of movements*. Edited by Roberts A. & Roberts B.L. Cambridge University Press. London.

Jordan, L.M., Brownstone, R.M. & Noga, B.R. (1992) Control of functional systems in the brainstem and spinal cord. *Curr. Op. Neurobiol.* **2**, 794-801.

Kaczmarek, L.K. & Levitan, I.B. (1987) *Neuromodulation: the biochemical control of neuronal excitability*. New York: Oxford University Press.

Kahn, J.A. & Roberts, A. (1982a) Experiments on the central pattern generator for swimming in embryos of the amphibian *Xenopus laevis*. *Phil. Trans. Roy. Soc. B.* **296**, 229-243.

Kahn, J.A. & Roberts, A. (1982b) The neuromuscular basis of rhythmic struggling movements in embryos of *Xenopus laevis*. *J. Exp. Biol.* **99**, 197-203.

Katakura, N. & Chandler, S.H. (1990) An iontophoretic analysis of pharmacologic mechanisms responsible for trigeminal motoneuronal discharge during masticatory-like activity in the guinea pig. *J. Neurophysiol.* **63**, 356-369.

Keef, K.D., Murray, D.C., Sanders, K.M. & Smith, T.K. (1997) Basal release of nitric oxide induces an oscillatory motor pattern in canine colon. *J. Physiol. (Lond)*. **499**, 773-786.

Keihn, O., Hounsgaard, J. & Sillar, K.T. (1997) Basic building blocks of vertebrate spinal central pattern generators. In: *Neurons, Networks and Motor*

Behaviour. Eds. P.S.G. Stein, S. Grillner, A.I. Selverston & D.G. Stuart. Cambridge: MIT Press. pp. 47-59.

Kessler, J.A. & Black, I.B. (1982) Regulation of substance P in adult rat sympathetic ganglia. *Brain Res.* **234**, 182-187.

Kiehn, O. & Kjaerulff, O. (1996) Spatiotemporal characteristics of 5-HT and dopamine-induced hindlimb locomotor activity in the in vitro neonatal rat. *J. Neurophysiol.* **75**, 1472-1482.

Kiehn, O., Hultborn, H. & Conway, B.A. (1992) Spinal locomotor-activity in acutely spinalized cats induced by intrathecal application of noradrenaline. *Neurosci. Lett.* **143**, 243-246.

Kitazawa, T., Saito, K. & Ohga, A. (1985) Effects of catecholamines on spinal motoneurons and spinal reflex discharges in the isolated spinal cord of the newborn rat. *Dev. Brain Res.* **19**, 31-36.

Kjaerulff, O. & Kiehn, O. (1997) Crossed rhythmic synaptic input to motoneurons during selective activation of the contralateral spinal locomotor network. *J. Neurosci.* **17**, 9433-9447.

Kloas, W., Reinecke, M. & Hanke, W. (1997) Stage-dependent changes in adrenal steroids and catecholamines during development in *Xenopus laevis*. *Gen. Comp. Endocrinology.* **108**, 416-426.

Kondo, S. & Marty, A. (1997) Portion kinase A-mediated enhancement of miniature IPSC frequency by noradrenaline in the rat cerebellar stellate cells. *J. Physiol.* **498**, 165-176.

Kondo, S. & Marty, A. (1998) Differential effects of noradrenaline on the evoked, spontaneous and miniature IPSCs in the rat cerebellar stellate cells. *J. Physiol.* **509**, 233-243.

Kudo, N. & Yamada, T. (1987) *N*-methyl-D,L-aspartate-induced locomotor activity in a spinal cord hind-limb muscles preparation of the newborn rat studied in vitro. *Neurosci. Lett.* **75**, 43-48.

Kuraishi, Y., Hirota, N., Sato, Y., Hino, Y., Sato, M. & Takagi, H. (1985) Noradrenergic inhibition of the release of substance P from the primary afferents in the rabbit dorsal horn. *Brain Res.* **381**, 266-274.

Kuwada, J.Y. (1986) Cell recognition by neuronal growth cones in a simple vertebrate embryo. *Science.* **233**, 740-746.

Langley, J.N. (1901) Observations on the physiological action of extracts of the supra-renal bodies. *J.Physiol. (Lond).* **27**, 237-256.

Larkman, P.M. & Kelly J.S. (1996) Characterisation of the 5-HT-sensitive and noradrenaline-sensitive potassium conductance in neonatal rat facial motoneurons in-vitro. *J. Physiol. (Lond).* **495P**, P 55.

Lincoln, T.M. & Cornwell, T.L. (1993) Intracellular cyclic-GMP receptor proteins. *FASEB J.* **7**, 328-338.

Llano, I. & Gerschenfeld, H.M. (1993) β -adrenergic enhancement of inhibitory synaptic activity in rat cerebellar slices. *J. Physiol.* **486**, 163-176.

Lundberg, J.M., Terenius, L., Hökfelt, T. & Goldstein, M. (1983) High levels of neuropeptide Y in peripheral noradrenergic neurones in various mammals including man. *Neurosci. Lett.* **42**, 138-150.

Lyons, W.E., Fritschy, J.M. & Grzanna, R. (1989) The noradrenergic neurotoxin DSP-4 eliminates the ceruleospinal projection but spares projections of the A5 and A7 groups to the ventral horn of the rat spinal-cord. *J. Neurosci.*, **9**, 1481-1489.

McCall, R.B. & Aghajanian, G.K. (1979) Serotonergic facilitation of facial motoneurone excitation. *Brain Res.* **169**, 11-27.

McDearmid, J.R., Scrymgeour-Wedderburn, J.F. & Sillar, K.T. (1997) Aminergic modulation of glycine release in a spinal network controlling swimming in *Xenopus laevis*. *J. Physiol.* **503**, 111-117.

McPherson, D.R. & Kemnitz, C.P. (1994) Modulation of lamprey fictive swimming and motoneuron physiology by dopamine, and its immunocytochemical localisation in the spinal-cord. *Neurosci. Lett.* **166**, 23-26.

McPherson, D.R., Buchanan, J.T. & Kasicki, S. (1994) Effects of strychnine on fictive swimming in the lamprey -evidence for glycinergic inhibition discrepancies with model predictions, and novel modulatory rhythms. *J. Comp. Physiol. A.* **175**, 311-321.

Marshall, K.C. & Engberg, I. (1979) Reversal potential for nordrenaline-induced hyperpolarisation of spinal motoneurons. *Science*. **205**, 422-424.

Millar, J. & Williams, G.V. (1989) Effects of iontophoresis of noradrenaline and stimulation of the periaqueductal gray on single-unit activity in the rat superficial dorsal horn. *J. Comp. Neurol.* **287**, 119-133.

Mintz, I., Gotow, T., Triller, A. & Korn, H. (1989) Effect of serotonergic afferents on quantal release at central inhibitory synapses. *Science*. **245**, 190-192.

Neuman, R.S. (1985) Action of serotonin and norepinephrine on spinal motoneurons following blockade of synaptic transmission. *Can. J. Physiol. Pharmacol.* **63**, 735-738.

Nieuwkoop, P.D. & Faber, J. (1956) *Normal tables for Xenopus laevis (Daudin)*. Amsterdam: North Holland.

Noga, B.R., Cowley, K.C., Huang, A., Jordan, L.M. & Schmidt, B.J. (1993) Effects of inhibitory amino acid antagonists on locomotor rhythm in the decerebrate cat. *Soc. Neurosci. Abst.* **19**, 540.

Nygren, L.G. & Olsen, L. (1977) A new major projection from the locus coeruleus: the major source of noradrenergic nerve terminals in the ventral and dorsal columns of the spinal cord. *Brain Res.* **131**, 85-93.

Odell, T.J., Hawkins, R.D., Kandel, E.R. & Arancio, O. (1991) Tests of the roles of 2 diffusible substances in long-term potentiation -evidence for nitric-oxide as a possible early retrograde messenger. *Proc. Nat. Acad. Sci. USA*. **88**, 11285-11289.

Ono, H., Hasebe, Y., Satoh, M., Nagao, T., Ohta, S., Hirobe, M. & Fukuda, H. (1991) Amphetamine-antagonistic properties of 4-phenyl-1,2,3,4-tetrahydroisoquinoline -inhibition of spinal reflex-enhancing effects of methamphetamine, phenylethylamine and nomifensine. *Brain Res.* **564**, 319-322.

Ono, H., Fukushima, C. & Fukuda, H. (1993) Effect of the centrally acting muscle-relaxant tizanidine on spinal reflexes -involvement of descending noradrenergic systems. *Jap. J. Pharmacol.* **62**, 357-362.

Oliver, G. & Schäfer, E.A. (1894) On the physiological action of extract of the suprarenal gland. *J. Physiol. (Lond)*. **16**, 1.

Oliver, G. & Schäfer, E.A. (1895) The physiological effects of extracts of the suprarenal capsules. *J. Physiol. (Lond)*. **18**, 230-276.

O'Donnell, S.R. & Wanstall, J.C. (1982) Pharmacological experiments demonstrate that the toad (*Bufo marinus*) atrial beta-adrenoceptors are not identical with mammalian beta₂ or beta₁ adrenoceptors. *Life Sci.* **31**, 701-708.

O'Donovan, M.J. (1989) Motor-activity in the isolated spinal-cord of the chick-embryo -synaptic drive and firing pattern of single motoneurons *J. Neurosci.* **9**, 943-958.

O'Donovan, M., Sernagor, E., Sholomenko, G., Ho, S., Antal, M. & Yee, W. (1992) Development of spinal motor circuits in the chick embryo. *J. Exp. Zoology.* **261**, 261-273.

Oyama, Y., Akaike, N. & Carpenter, D.O. (1988) Strychnine decreases the voltage-dependent Ca^{2+} current of both *Aplysia* and frog ganglion neurons. *Cell. Mol. Neurobiol.* **8**, 307-314.

Palmer, R.M.J., Ferrige, A.G. & Moncada, S. (1987) Nitric oxide accounts for the biological activity of endothelium derived relaxing factor. *Nature.* **327**, 524-526.

Panchin, Y.V., Perrins, R.J. & Roberts, A. (1991) The action of acetylcholine on the locomotor central pattern generator for swimming in *Xenopus* embryos. *J. Exp. Biol.* **161**, 527-531.

Pang, I.H. & Vasko, M.R. (1986) Morphine and norepinephrine but not 5-hydroxytryptamine and γ -aminobutyric acid inhibit the potassium-stimulated release of substance P from rat spinal cord slices. *Brain Res.* **376**, 268-279.

Pearce, R.A. & Friesen, W.O. (1984) Intersegmental co-ordination of the leech swimming rhythm: comparison of in situ and isolated nerve cord activity with body wall movement *J. Neurophysiol.* **299**, 363-366.

Pearce, R.A. & Friesen, W.O. (1985) Intersegmental co-ordination of the leech swimming rhythm. 1. Roles of cycle period gradient and coupling strength. *J. Neurophysiol.* **54**, 1444-1459.

Perret, C. (1983) Centrally generated pattern of motoneurone activity during locomotion in the cat. In *Neural origin of rhythmic movements*. ed. A Roberts, B. Roberts. 405-422. *Brighton Symp. Soc. Exp. Biol.*

Perrins R. & Roberts A. (1995a) Cholinergic and electrical synapses between synergistic motoneurons in the *Xenopus laevis* embryo. *J Physiol.* **485** (1), 135-144.

Perrins R. & Roberts A. (1995b) Cholinergic and electrical motoneuron-to-motoneuron synapses contribute to on-cycle excitation during swimming in *Xenopus* embryos. *J. Neurophysiol.* **73**, 1005-1012.

Perrins R. & Roberts A. (1995c) Cholinergic contribution to excitation in a spinal locomotor central generator in *Xenopus* embryos. *J. Neurophysiol.* **73**, 1013-1019.

Perrins R. & Soffe, S.R. (1996a) Local-effects of glycinergic inhibition in the spinal-cord motor systems for swimming in amphibian embryos *J. Neurophysiol.* **76**, 1025-1035.

Perrins R. & Soffe, S.R. (1996b) Composition of excitatory drive during swimming in two amphibian embryos: *Rana* and *Bufo*. *J. Comp. Physiol. A.* **179**, 563-573.

Phillis, J., Tebecis, A.K. & York, D.H. (1968) Depression of spinal motoneurons by noradrenaline, 5-hydroxytryptamine and histamine. *Eur. J. Pharmacol.* **4**, 471-475.

Poon, M. (1980) Induction of swimming in lamprey by L-DOPA amino acids. *J. Comp. Physiol.* **136**, 337-344.

Rajfer, J., Aronson, W.J., Bush, P.A., Dorey, F.J. & Ignarro, L.J. (1992) Nitric-oxide as a mediator of relaxation of the corpus cavernosum -reply. *New Eng. J. Med.*, **326**, 1638.

Rajaofetra, N., Poulat, P., Marlier, L., Geffard, M., Privat, A. (1992) Prenatal and postnatal-development of noradrenergic projections to the rat spinal-cord - an immunocytochemical study. *Dev. B. Res.* **67**, 237-246.

Raeymaekers, L., Hofmann, F & Casteels, R. (1988) Cyclic GMP-dependent protein-kinase phosphorylates phospholamban in isolated sarcoplasmic-reticulum from cardiac and smooth-muscle. *Biochem. J.* **252**, 269-273.

Reddy, V.K., Fung, S.J., Zhuo, H. & Barnes, C.D. (1989) Spinally projecting noradrenergic neurons of the dorsolateral pontine tegmentum - a combined immunocytochemical and retrograde labelling study *B. Res.* **491**, 144-149.

Reith, C.A. (1996) GABAergic modulation of locomotor rhythmically during post-embryonic development in *Xenopus laevis*. PhD thesis. University of St.Andrews.

Reith, C.A. & Sillar, K.T. (1997) Pre- and postsynaptic modulation of spinal GABAergic neurotransmission by the neurosteroid, 5 β -pregnan-3 α -ol-20-one. *Brain Res.* **770**, 202-212.

Roberts, A. (1978) Pineal eye and behaviour in *Xenopus* tadpoles. *Nature.* **273**, 774-775.

Roberts, A. (1990) How does a nervous system produce behaviour? A case study in neurobiology. *Sci. Prog. Oxf.* **74**, 31-35.

Roberts, A. (1996) Trigeminal pathway for the skin impulse to initiate swimming in hatchling *Xenopus* embryos. *J. Physiol. (Lond.)* **493P**, P40-P41.

Roberts, A. & Clarke, J.D.W. (1982) The neuroanatomy of an amphibian embryo spinal cord. *Phil. Trans. R. Soc. Lond. B.* **296**, 195-212.

Roberts, A. & Hayes, B.P. (1977) The anatomy and function of 'free' nerve endings in an amphibian skin sensory system. *Proc. Roy. Soc., Lond. B.* **196**, 415-429.

Roberts A. & Kahn A.J. (1982) Intracellular recordings from spinal neurons during 'swimming' in paralysed amphibian embryos. *Phil. Trans. Roy. Soc. B.* **296**, 213-228.

Roberts, A. & Smyth, D. (1974) The development of dual touch sensory systems in embryos of the amphibian, *Xenopus laevis*. *J. Comp. Physiol.* **88**, 31-42.

Roberts, A. & Tunstall, M.J. (1990) Mutual re-excitation with post-inhibitory rebound: a simulation study on the mechanisms for locomotor rhythm generation in the spinal cord of *Xenopus* embryos. *Eur. J. Neurosci.* **2**, 11-23.

Roberts, A., Dale, N. Evoy, W.H. & Soffe, S.R. (1985) Synaptic potentials in motoneurons during fictive swimming in spinal *Xenopus* embryos. *J. Neurophysiol.* **54**, 1-10.

Roberts, A., Soffe, S. & Dale, N. (1986) Spinal interneurons and swimming in frog embryos. In: *Neurobiology of Vertebrate locomotion*. Eds. S. Grillner, R. Herman, P.G.S. Stein & D. Stuart. Macmillian Lond.

Roberts, A., Dale, N., Ottersen, O.P. & Storm-Mathisen, J. (1987) The early development of neurons with GABA immunoreactivity in the CNS of *Xenopus laevis* embryos. *J. Neurophysiol.* **261**, 435-449.

Roberts, A., Dale, N., Ottersen, O.P. & Storm-Mathisen, J. (1988) Development and characterisation of commissural interneurons in the spinal cord of *Xenopus laevis* embryos revealed by antibodies to glycine. *Development.* **103**, 447-461.

Roberts, A., Tunstall, M.J. & Wolf, E. (1995) Properties of networks controlling locomotion and significance of voltage dependency of NMDA channels: simulation study of rhythm generation sustained by positive feedback. *J. Neurophysiol.* **73**, 485-495.

Sas, E., Maler, L. & Tinner, B. (1990) Catecholaminergic systems in the brain of a gymnotiform teleost fish: An immunohistochemical study. *J. Comp. Neurol.* **292**, 127-162.

Schaad, N.C., Vanecek, J., Kosar, E., Aubry, J.M. & Schulz, P.E. (1995) Adrenergic control of rat pineal NO synthase. *J. Neurochem.* **65**, 935-993.

Schuman, E.M. & Madison, D.V. (1991) A requirement for the intercellular messenger nitric-oxide in long-term potentiation. *Science.* **254**, 1503-1506.

Schwarz, P., Diem, R., Dun, N.J. & Fostermann, U. (1995) Endogenous and exogenous nitric oxide inhibits norepinephrine release from rat-heart sympathetic nerves. *Circ. Res.* **77**, 841-848.

Schwarz, P.M., Rodriguez-Pasual, F., Koesling, D., Torres, M. & Fostermann, U. (1998) Functional coupling of nitric oxide synthase and soluble guanylyl cyclase in controlling catecholamine secretion from bovine chromaffin cells. *Neurosci.* **82**, 255-265.

Scrymgeour-Wedderburn, J.F.S., Reith, C.A. & Sillar, K.T. (1997) Voltage oscillations in *Xenopus* spinal cord neurones: developmental onset and dependence on co-activation of NMDA and 5-HT receptors. *Eur. J. Neurosci.* **9**, 1473-1482.

Seilicovich, A., Lasaga, M., Befumo, M., Duvilanski, B.H., Diaz, M.D., Rettori, V. & McCann, S.M. (1995) Nitric-oxide inhibits the release of norepinephrine and dopamine from the medial basal hypothalamus of the rat. *Proc. Nat. Acad. Sci. U.S.A.* **92**, 11299-11302.

Shupliakov, O., Pierbone, V.A., Gad, H. & Brodin, L. (1995) Synaptic vesicle depletion in reticulospinal axons is reduced by 5-hydroxytryptamine: Direct evidence for presynaptic modulation of glutamatergic transmission. *Eu. J. Neurosci.* **7**, 1111-1116.

Sillar, K.T. & Soffe, S.R. (1989) Locomotion based on rhythmic motor bursts in *Rana-temporaria* embryos. *J. Physiol.* **412**, 76.

Sillar, K.T. & Roberts, A. (1992a) Phase-dependent modulation of a cutaneous sensory pathway by glycinergic inhibition from the locomotor rhythm generator. *Euro. J. Neurosci.* **4**, 1022-1034.

Sillar, K.T. & Roberts, A. (1992b) The role of premotor interneurons in phase dependent modulation of a cutaneous reflex during swimming in *Xenopus laevis* embryos. *J. Neurosci.* **12**(5), 1647-1657.

Sillar, K.T. & Roberts, A. (1993) Control of frequency during swimming in *Xenopus* embryos: a study on interneuronal recruitment in a spinal rhythm generator. *J. Physiol.* **472**, 557-572.

Sillar, K. T. & Wedderburn, J. F. S. (1993) Development of mechanisms for rostrocaudal phase-delay in fictive swimming of postembryonic *Xenopus-laevis* larvae. *J. Physiol. (Lond)*. **459**, P 11.

Sillar, K. T., Wedderburn, J. F. S. & Simmers, A. J. (1991) The post-embryonic development of locomotor rhythmicity in *Xenopus laevis* tadpoles. *Proc. R. Soc. (Lond)*. **246**, 147-153.

Sillar, K.T. & Woolston, A.M. (1995) A dual mechanism for serotonergic modulation of *Xenopus* swimming: spinal inhibition and brainstem excitation. *Soc. Neurosci. Abst.* **21**, 167.

Sillar, K. T., Simmers, A. J. & Wedderburn, J. F. S. (1992) The post-embryonic development of cell properties and synaptic drive underlying locomotor rhythm generation in *Xenopus* larvae. *Proc. Roy. Soc. Lond. B.*, **249**, 65-70.

Sillar, K. T., Wedderburn, J. F. S. & Simmers, A. J. (1992) Modulation of swimming rhythmicity by 5-hydroxytryptamine during post-embryonic development in *Xenopus laevis*. *Proc. Roy. Soc. Lond. B.* **250**, 107-114.

Sillar, K.T., Woolston, A.M. & Wedderburn, J.F.S. (1992) Development and role of serotonergic innervation to the spinal-cord in hatchling *Rana-temporaria* and *Xenopus-laevis* tadpoles. *J. Physiol.(Lond)*. **446**, P 323.

Sillar, K.T., Woolston, A.M. & Wedderburn, J.F.S. (1995) Involvement of brain-stem serotonergic interneurons in the development of a vertebrate spinal locomotor circuit. *Proc Roy Soc Lond.* **259**, 65-70.

Sillar, K.T., Reith, C.A. & McDermid, J. The deveopment and aminergic modulation of a spinal network controlling swimming in *Xenopus* larvae. *Annals. New York Acad. Sci.* (In press).

Smeets, W.J.A.J. & Steinbusch, H.W.M. (1989) Distribution of noradrenaline immunoreactivity in the forebrain and midbrain of the lizard *Gekko gekko*. *J. Comp. Neurol.* **285**, 453-466.

Smeets, W.J.A.J. & Steinbusch, H.W.M. (1990) New insights into the reptilian catecholaminergic systems as revealed by antibodies against the neurotransmitters and their synthetic enzymes. *J. Chem. Neuroanat.* **3**, 25-43.

Smith, J.C. & Feldman, J.L. (1987) In vitro brainstem-spinal cord preparation for the study of motor systems for mammalian respiration and locomotion. *J. Neurosci. Methods.* **21**, 321-333.

Smith, J.C., Feldman, J.L. & Schmidt, B.J. (1988) Neural mechanisms generating locomotion studied in mammalian brainstem- spinal cord in vitro. *FASEB J.* **2**, 2283-2288.

Soffe, S.R. (1987) Ionic and pharmacological properties of reciprocal inhibition in *Xenopus* embryo motoneurons. *J. Physiol.* **382**, 463-473.

Soffe, S.R. (1989) Roles of glycinergic inhibition and N-methyl-D-aspartate receptor mediated excitation in the locomotor rhythmicity of one half of the *Xenopus* embryo CNS. *Eur. J. Neurosci.* **1**, 561-571.

Soffe, S.R. (1990) Active and passive membrane properties of spinal cord neurons which are rhythmically active during swimming in *Xenopus* embryos. *Euro. J. Neurosci.* **2**, 1-10.

Soffe, S.R. (1991a) Centrally generated rhythmic and non-rhythmic behavioural responses in *Rana temporaria* embryos. *J. exp. Biol.* **156**, 81-99.

Soffe, S.R. (1991b) Triggering and gating of motor responses by sensory stimulation: behavioural selection in *Xenopus* embryos. *Proc. R. Soc. Lond. B.* **246**, 197-203.

Soffe, S.R. (1993) Two distinct motor patterns are driven by common premotor and motor neurons in a simple vertebrate spinal cord. *J. Neurosci.* **13**, 4456-4469.

Soffe, S.R. (1996) Motor patterns for two distinct rhythmic behaviours evoked by excitatory amino acid agonists in the *Xenopus* embryo spinal cord. *J. Neurophysiol.* **75**, 1815-1825.

Soffe, S.R. & Perrins, R. (1997) Neuronal firing properties and swimming motor patterns in young tadpoles of four amphibians: *Xenopus*, *Rana*, *Bufo* and *Triturus*. *J. Comp. Physiol.* **181**, 71-81.

Soffe, S.R. & Roberts, A. (1982a) Tonic and phasic synaptic input to spinal cord motoneurons during fictive locomotion in frog embryos. *J. Neurophysiol.* **48**, 1279-1288.

Soffe, S.R. & Roberts, A. (1982b) Activity of myotomal motoneurons during fictive swimming in frog embryos. *J. Neurophysiol.* **48**, 1274-1278.

Soffe, S.R. & Sillar, K.T. (1991) Patterns of synaptic drive to ventrally located spinal neurons in *Rana temporaria* embryos during rhythmic and non-rhythmic motor responses. *J. Exp. Biol.* **156**, 101-118.

Soffe, S.R., Clarke, J.D.W. & Roberts, A. (1984) Activity of commissural interneurons in the spinal cord of *Xenopus* embryos. *J. Neurophysiol.* **51**, 1247-1267.

Soffe, S.R., Clarke, J.D.W. & Roberts, A. (1983) Swimming and other centrally generated motor patterns in newt embryos. *J. comp. Physiol.* **152**, 535-544.

Springall, D.R., Riverosmoreno, V., Buttery, L., Suburo, A., Bishop, A.E. Merrett, M., Moncada, S. & Polak, J.M. (1992) Immunological detection of nitric-oxide synthase(s) in human tissues using heterologous antibodies suggesting different isoforms. *Histochem.* **98**, 259-266.

Sqalli-Housaini, Y., Cazalets, J.R., Martini, F. & Clarac, F. (1993) Induction of fictive locomotion by sulphur containing amino acids in an in vitro newborn preparation. *Eur. J. Neurosci.* **5**, 1226-1232.

Steeves, J.D., Schmidt, B.J., Skovgaard, B.J. & Jordan, L.M. (1980) The effect of noradrenaline and 5-hydroxytryptamine depletion on locomotion in the cat. *Brain Res.* **185**, 349-362.

Stehle, R.L. & Ellsworth, H.C. (1937) Dihydroxyphenylethanolamine (arterenol) as a possible sympathetic hormone. *J. Pharmacol. Exp. Ther.*, **59**, 114-121.

Steinbusch, H.W.M. (1981) Distribution of serotonin immunoreactivity in the central nervous system of the rat- cell bodies and terminals. *Neuroscience.* **6**, 557-618.

Stevens, R.T., Hodge, C.J., & Apkarian, A.V. (1982) Köelliker-Fuse nucleus: the principal source of pontine catecholaminergic cells projecting to the lumbar spinal cord of the cat. *Brain Res.* **239**, 589-594.

Stewart, J.E., Barbeau, H. & Gauthier, S. (1991) Modulation of locomotor patterns and spasticity with clonidine in spinal-cord injured patients. *Can. J. Neurol. Sci.* **18**, 321-332.

Stewart, T.L., Michel, A.D., Black, M.D. & Humphrey P.P.A., (1996) Evidence that nitric-oxide causes calcium-independent release of [H-3] dopamine from rat striatum in vitro. *J. Neurochem.* **66**, 131-137.

Strahlendorf, J.C., Strahlendorf, H.K., Kingsley, R.E., Gintautas, J. & Barnes, C.D. (1980) Facilitation of lumbar monosynaptic reflexes by locus coeruleus stimulation. *Neuropharm.* **19**, 255-230.

Sun, Q.Q. & Dale, N. (1998) Developmental changes in expression of ion currents accompany maturation of locomotor pattern in frog tadpoles. *J. Physiol.* **507**, 257-264.

Takemine, J. (1901) The isolation of the active principle of the suprarenal gland. *J. Physiol.(Lond)*. **27**, 30-39P.

Tanaka, H., Takahashi, S., Miyamoto, A., Oki, J., Cho, K., Okuno, A. (1996) Developmental-changes in the noradrenergic innervations of spinal motoneurons in neonatal rats. *Pediatric Neurol.* **14**, 21-27.

Tégner, J., Matsushima, T.M., El Manira, A., & Grillner, S. (1993) The spinal GABA system modulates burst frequency and intersegmental co-ordination in the lamprey: differential effects of GABA_A and GABA_B receptors. *J. Neurophysiol.* **69**, 647-657.

Terenghi, G., Riverosmoreno, V., Hudson, L.D., Ibrahim, N.B.N. & Polak, J.M. (1993) Immunohistochemistry of nitric-oxide synthase demonstrates immunoreactive neurons in spinal-cord and dorsal-root ganglia of man and rat. *J. Neurol. Sci.* **118**, 34-37.

Thatikunta, P., Chakder, S. & Rattan, S. (1993) Nitric-oxide synthase inhibitor inhibits catecholamine release caused by hypogastric sympathetic-nerve stimulation. *J. Pharm. Exp. Ther.* **267**, 1363-1368.

Tunstall, M.J. & Roberts, A. (1991) Longitudinal co-ordination of motor output during swimming in *Xenopus* embryos. *Proc. Roy. Soc. Lond. B.* **244**, 27-32.

Tunstall, M.J. & Roberts, A. (1994) A longitudinal gradient of synaptic drive in the spinal cord of *Xenopus* embryos and its role in co-ordination of swimming. *J. Physiol.* **474**, 393-405.

Tunstall, M.J. & Sillar, K., (1993) Physiological and developmental aspects of intersegmental co-ordination in *Xenopus* embryos and tadpoles. *Sem. Neurosci.* **5**, 29-40.

VanderMaelen, C.P. & Aghajanian, G.K. (1980) Intracellular studies showing modulation of facial motoneurone excitability by serotonin. *Nature (Lond).* **287**, 346-347.

VanderMaelen, C.P. & Aghajanian, G.K. (1982) Serotonin-induced depolarization of rat facial moto-neurons invivo -comparison with amino-acid transmitters. *Brain Res.* **239**, 139-152.

Vandongen, P.A.M., Hokfelt, T., Grillner, S., Verhofstad, A.A.J. & Steinbusch, H.W.M. (1985) Possible target neurons of 5-hydroxytryptamine fibers in the spinal-cord -immunohistochemistry combined with intracellular staining with lucifer yellow. *J. Comp. Neurol.* **234**, 523-535.

van Mier, P., Joosten, H.W.J., van Reden, R. & ten Donkelaar, H.J. (1986) The development of serotonergic raphespinal projections in *Xenopus laevis*. *Int. J. Dev. Neurosci.* **4**, 465-476.

Venturini, C.M., Plamer, R.M.J. & Moncada, S. (1993) Vascular smooth muscle contains a depletable store of vasodilator which is light-activated and resored by donors of nitric oxide. *J. Pharm. Exp. Ther.* **266**, 1497-1500.

Viala, D. & Buser, P. (1969) The effect of DOPA and 5-HTP on rhythmic efferent discharges in hindlimb nerves in the rabbit. *Brain Res.* **12**, 437-443.

Vogt, M. (1954) The concentration of sympathin in different parts of the central nervous system under normal conditions and after administration of drugs. *J. Physiol. (Lond)*. **154**, 52-67.

Wall, M.J. & Dale, N. (1993) GABA_B receptors modulate glycinergic inhibition and spike threshold in *Xenopus* embryo spinal neurons. *J. Physiol.* **469**, 275-290.

Wall, M.J. & Dale, N. (1994) A role for potassium currents in the generation of the swimming motor pattern of *Xenopus* embryos. *J. Neurophysiol.* **72**, 337-348.

Wallen, P. & Williams, T.L. (1984) Fictive locomotion in the lamprey spinal cord *in vitro* compared with swimming in the intact spinal animal. *J. Physiol. (Lond)*. **347**, 225-239.

Wedderburn, J.F.S. & Sillar, K.T. (1994) Modulation of rhythmic swimming activity in post-embryonic *Xenopus laevis* tadpoles by 5-hydroxytryptamine acting at 5HT_{1A} receptors. *Proc. R. Soc. Lond. B.* **257**, 59-66.

Weight, F.F. & Salmoirhagi, G.C. (1966) Responses of spinal cord interneurons to acetylcholine, norepinephrine and serotonin administered by microelectrophoresis. *J. Pharmacol. Exp. Ther.* **153**, 420-427.

Weight, F.F. & Salmoirhagi, G.C. (1967) Motoneurone depression by norepinephrine. *Nature*. **213**, 1229-1230.

Westlund, K.N. & Coulter, J.D. (1980) Descending projections of the locus coeruleus and subcoeruleus/medial parabrachial nuclei in monkey: axonal transport studies and dopamine- β -hydroxylase immunocytochemistry. *Brain Res. Rev.* **2**, 235-264.

Westlund, K.N., Bowker, R.M., Ziegler, M.G. & Coulter, J.D. (1981) Origins of spinal noradrenergic pathways demonstrated by retrograde transport of antibody to dopamine- β -hydroxylase. *Neurosci. Lett.* **25**, 243-249.

Westlund, K.N., Bowker, R.M., Ziegler, M.G. & Coulter, J.D. (1983) Noradrenergic projections to the spinal-cord of the rat. *B. Res.* **263**, 15-31.

Wheatley, M. & Stein, R.B. (1992) An in vitro preparation of the mud puppy for simultaneous intracellular and electromyographic recording during locomotion. *J. Neurosci.* **42**, 129-137.

White, S.R. & Neuman, R.S. (1980) Facilitation of spinal motoneurone excitability by 5-hydroxytryptamine and noradrenaline. *Brain Res.* **188**, 119-127.

White, S.R., Fung, S.J. & Barnes, C.D. (1991) Norepinephrine effects on spinal motoneurons. *Prog. Brain Res.* **88**, 343-350.

Wilson, S.P., Klein, R.L., Chang, K.J., Gasparis, M.S., Viveros, O.H. & Yang, W.H. (1980) Are opiod peptides co-transmitters in noradrenergic vesicles of sympathetic nerves? *Nature (Lond)*. **288**, 707-709.

Wohlberg, C.J., Davidoff, R.A. & Hackman J.C. (1986) Analysis of the responses of frog motoneurons to epinephrine and norepinephrine. *Neurosci. Lett.* **69**, 150-155.

Wolf, E. & Roberts, A. (1995) The influence of premotor populations on the frequency of spinal pattern generator for swimming in *Xenopus* embryos: a simulation study. *E. J. Neurosci.* **7**, 671-678.

Woolston, A-M., Wedderburn, J.F.S. & Sillar, K.T. (1994) Descending serotonergic spinal projections and modulation of locomotor rhythmicity in *Rana temporaria*. *Proc. Roy. Soc. B.* **255**, 73-79.

Yamamoto, R., Wada, A., Asada, Y., Yanagita, T., Yuhi, T., Niina, H., Sumiyoshi, A., Kobayashi, H. & Lee, T.J.F. (1997) Nitric oxide-dependent and -independent norepinephrine release from rat mesenteric arteries. *Am. J. Phys. - Heart Circ. Phys.* **41**, H207-H210.

Yoshida, Y., Sun, H.T., Cai, J.Q. & Imai, S. (1991) Cyclic GMP-dependent protein-kinase stimulates the plasma-membrane Ca²⁺ pump ATPase of vascular smooth-muscle via phosphorylation of a 240-kDa protein. *J. Biol. Chem.* **266**, 19819-19825.

Yawo, H. (1996) Noradrenaline modulates transmitter release by enhancing the Ca^{2+} sensitivity of exocytosis in the chick ciliary presynaptic terminal. *J. Physiol.* **493**, 385-391.

Publications arising from this work.

Full papers

McDearmid, J.R., Scrymgeour-Wedderburn, J.F. & Sillar, K.T. (1997) Aminergic modulation of glycine release in a spinal network controlling swimming in *Xenopus laevis*. *Journal of Physiology*, **503** (1), 111-117.

Published Abstracts

McDearmid, J.R. & Sillar, K.T. (1997) A slow non-rhythmic motor pattern elicited by both noradrenaline and nitric oxide in embryos of the frog *Rana temporaria*. In. *Journal of Physiology*, **504**, 12P.

Book Chapters

Sillar, K.T., Reith, C.A. & McDermid, J. The development and aminergic modulation of a spinal network controlling swimming in *Xenopus* larvae. *Annals. New York Acad. Sci.* (In press).